AD_____

Award Number: DAMD17-00-1-0208
               DAMD17-00-1-0207

TITLE: Synthetic Lethality in Breast Cancer Cells: Genes
        Required for Tumor Survival

PRINCIPAL INVESTIGATOR: Gregory J. Hannon, Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
                            Cold Spring Harbor, New York 11724

REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                         Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

		Form Approved OMB No. 074-0188

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Jun 01 – 1 Jun 02)	

4. TITLE AND SUBTITLE Synthetic Lethality in Breast Cancer Cells: Genes Required for Tumor Survival	5. FUNDING NUMBERS DAMD17-00-1-0208 DAMD17-00-1-0207

6. AUTHOR(S)
Gregory J. Hannon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cold Spring Harbor Laboratory Cold Spring Harbor, New York 11724 E-Mail: hannon@cshl.edu	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited	12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)

The conversion of a normal cell into a cancer cell proceeds through a series of genetic and epigenetic alterations. We have proposed to use well-established genetic methodologies to identify novel anti-cancer targets via their specific, genetic interactions with common cancer mutations. In short, we will identify genetic alterations that are neutral in normal cells, but that are lethal when combined with cancer mutations. This "synthetic lethality" approach may identify potential therapeutic targets that are highly specific to the cancer cell. In the past year, we have made substantial progress toward the goal of developing technologies necessary to making this type of target search a reality. We have devised genetically defined human cancer models that can be used to conduct synthetic lethality screens and have developed new methodologies for manipulating gene expression in mammalian cells.

14. SUBJECT TERMS breast cancer, cancer biology, genetics, synthetic lethality, apoptosis	15. NUMBER OF PAGES 96
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Introduction

My laboratory has long been dedicated to the application of genetic principles to both the understanding of cancer biology and the search for novel anticancer targets. Since the 1970s, the war on cancer has been based on the notion that studying the disease will lead to the discovery of vulnerabilities, which can be exploited in the clinic. While many underlying genetic determinants of cancer have been identified, this knowledge has failed to translate into new therapeutic strategies, with only a handful of exceptions. One hypothesis is that this failure has largely been due to the genetically intractable nature of cultured mammalian cells. In part due to work from my laboratory, dsRNA-induced gene silencing, or RNA interference (RNAi), in mammalian systems has emerged as a tool that is likely to re-invigorate the field of somatic cell genetics and in the process revolutionize the study of human disease. During the past year, we have reached a point at which any gene in the human genome can conceivably be targeted using small dsRNA gene silencing triggers – *small interfering RNAs (siRNAs) or expressed short hairpin RNAs (shRNAs)*. I have recently received an Innovator award from this same program that will fund the construction of shRNA-expression cassettes on a large, and possibly whole-genome scale. We will be able to use these tools within the present programs to test the notion that synthetic lethality is a plausible approach to the discovery of novel anticancer targets. I note that the goals of this grant and that of the Innovator award are distinct. The innovator award is to develop high-throughput procedures to create a resource for the entire cancer community. This program will apply that resource to a very specific problem in cancer biology.

Body

Genetically defined models of human cancer :

One of the long-standing interests in our lab is the elucidation of the minimum genetic requirements for the transformation of human cells. Utilizing the technique of retroviral gene transduction, we have previously shown that normal human fibroblasts could be transformed with the oncogene combination of adenovirus E1A, MDM2, and H-RasV12. This transformation model is significantly different from those previously published in that there is a distinct absence of telomerase expression, either through direct introduction of the catalytic subunit hTERT or by an oncogene capable of activating hTERT. Analysis of telomerase status in these engineered cells and the resultant primary tumors indicates that telomerase is not activated, indicating that telomerase activity is not essential in the initial transformation event.

Upon explantation into culture, however, cells derived from these primary tumors undergo widespread apoptosis and senescence, phenomena indicative of telomere crisis, although a small percentage of explanted cells are able to survive this crisis event. Interestingly enough, the cells that survive after

4

explantation are telomerase positive. When these cells are injected into a second nude mouse, the kinetics of tumor formation are similar to those of the primary tumors, indicating that no additional genetic alterations were acquired *in vivo* or *in vitro* other than telomerase activation. It is interesting to note that telomerase activation does not appear to enhance tumorigenicity, but serves as an enabling event to prevent telomere shortening and widespread genomic instability characteristic of cells undergoing mitotic crisis.

Through our studies utilizing this E1A-based system, we have begun to elucidate the minimum genetic alterations that must occur to transform a normal human cell into one that is cancerous. Currently, we are focusing on taking this tractable transformation system and adapting it for use in an epithelial cell system to more accurately model events that lead to tumorigenesis. In addition, we are also continuing to make progress on the development a human cell transformation model comprised entirely of cellular oncogenes.

A manuscript describing these results is under review and is attached.
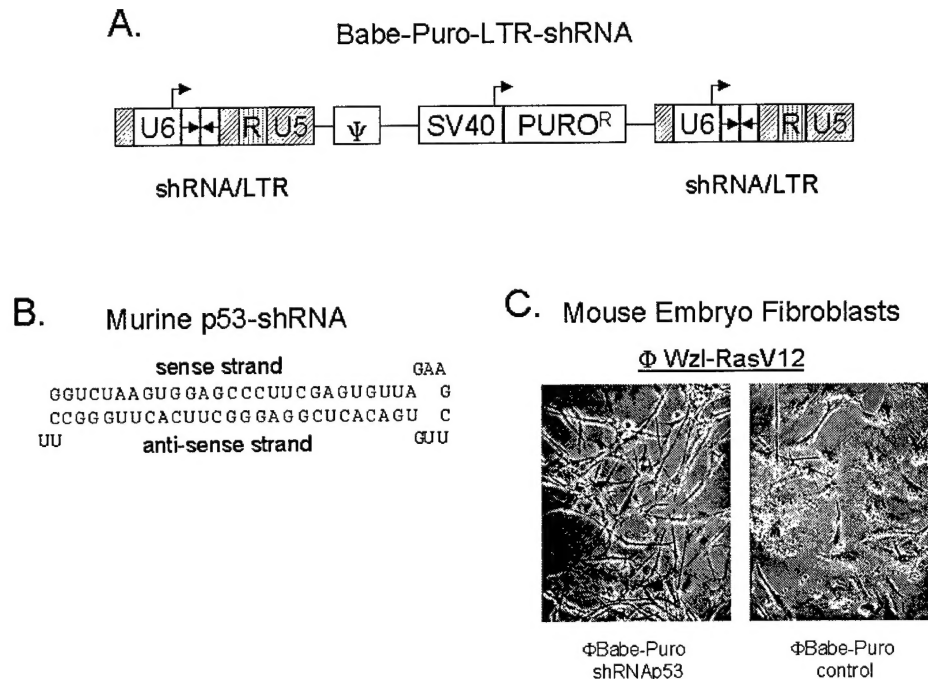
Loss of function genetics

Over the last year, we have settled on the strategy of expressing short hairpin RNAs for doing genetic screens in mammalian cells. After extensive comparisons of chemically synthesized siRNAs and shRNAs, we feel that shRNAs are at least as good, if not superior to, siRNAs. In addition, shRNAs have the added benefit of being deliverable using a wide variety of reagents and well-worn strategies for gene transfer. We have tested a number of strategies for shRNA expression (including different promoters, H1 and U6, different structural elements, different stem lengths etc.) While these studies are still ongoing, we have settled on the U6 promoter and stem lengths of either 19 or 29 nt. The decision between these two will be taken shortly and will be based on tests for off-target effects using microarrays.

A manuscript describing these findings was published earlier this year and is attached.

Furthermore, we have found that shRNA expression vectors are functional in living mice. This provides a potential template for the use of RNAi as a gene therapeutic. A manuscript describing these findings is attached.

We continue to develop strategies for delivering shRNA expression cassettes to cells in an efficient manner. Specifically, we have tested several retroviral backbones, and have identified combinations of elements that can efficiently deliver functional shRNA expression cassettes into the genome (see figure 1). Specifically, we have MoMuLV viruses and MSCV viruses that induce potent and stable suppression of target genes such as p16 INK4A and p53.

5

A. Babe-Puro-LTR-shRNA

shRNA/LTR   shRNA/LTR

B. Murine p53-shRNA

sense strand   GAA
GGUCUAAGUGGAGCCCUUCGAGUGUUA G
CCGGGUUCACUUCGGGAGGCUCACAGU C
UU   anti-sense strand   GUU

C. Mouse Embryo Fibroblasts

Φ Wzl-RasV12

ΦBabe-Puro   ΦBabe-Puro
shRNAp53   control

Figure 1: Stable expression of an shRNA using a retroviral system. This figure demonstrates one strategy for expressing shRNAs from retroviruses. A U6-p53-shRNA was inserted into the 3' LTR of a MoMuLV pBabe-Puro retroviral construct. **A.** Shows the predicted structure and orientation of the retrovirus as integrated into the genome of the infected cell. **B.** The predicted structure of a murine p53 shRNA. **C.** An assay for bypass of rasV12 induced senescence in early passage mouse embryo fibroblasts (P2). Cells were first transduced with Babe-Puro alone or Babe-Puro-LTR-U6-p53-shRNA and selected in puromycin for 3 days, after which cells were infected with Wzl-Hygro-rasV12 and treated with hygromycin. Only cells initially receiving Babe-Puro-LTR-p53-shRNA were morphologically transformed by rasV12 and continued to divide (see text). A time point 5 days after transduction with rasV12 is shown. Potential complications with this strategy would arise if shRNAs target viral and drug resistance transcripts in packaging or target cells, or if shRNAs target essential genes in the packaging cells. Thereby we are currently designing inducible U6 constructs for expression from self-inactivating retroviruses.

In short, I now feel that we are poised to move forward with loss-of-function, genetic screens in our defined models of human cancer cells. A manuscript (review article) describing the overall strategy and implications is in press at Cancer Cell and is attached.

Key Research Accomplishments

- Transformation of normal human cells in the absence of telomerase activation or other telomere maintenance strategies

6

- Discovery that short hairpin RNAs (shRNAs) can trigger stable gene silencing by RNAi in mammalian cells

- Discovery that shRNAs are effective in living animals (see attached manuscript)

- Design of efficient delivery strategies for shRNA expression.

Reportable outcomes

Manuscripts (4)
- Seger et al (submitted)
- Paddison et al., (Genes and Development, attached)
- Paddison et al., (Cancer Cell in press, attached)
- McCaffrey et al., (Nature, in press, attached)

Reagents
- shRNA expression vectors
- A web site for shRNA and siRNA design (http://www.cshl.org/public/SCIENCE/hannon.html)
- shRNA delivery systems (retroviruses)

Conclusions

Over the past year, I feel that we have made tremendous strides in the design of strategies that will permit loss-of-function screens for synthetic lethals in mammalian cells.

Transformation of normal human cells in the absence of telomerase activation

Yvette R. Seger[1,2], Marta García-Cao[3], Sara Piccinin[4], Crucifissa Lo Cunsolo[5], Claudio Doglioni[5], María A. Blasco[4], and Gregory J. Hannon[1,6], and Roberta Maestro[4]

1 Cold Spring Harbor Laboratory
1 Bungtown Road
Cold Spring Harbor, NY 11724

2 Graduate Program in Genetics
State University of New York at Stony Brook
Stony Brook, NY 11794

3 Department of Immunology and Oncology
Centro Nacional de Biotecnología-CSIC
Campus Cantoblanco
28049 Madrid, SPAIN

4 MMNP Unit - Experimental Oncology
CRO, National Cancer Institute
Via Pedemontana Occ. 12
33081 Aviano (PN) ITALY

5 Department of Histopathology,
Belluno City Hospital,
32100 Belluno, ITALY

6 To whom correspondence should be addressed :
Phone : 516-367-8889
Fax : 516-367-8874
hannon@cshl.org

Summary

Our knowledge of the transformation process has emerged largely from studies of primary rodent cells and animal models. However, numerous attempts to transform human cells using oncogene combinations that are effective in rodents have proven unsuccessful. These findings strongly argue for the study of homologous experimental systems. Here we report that the combined expression of adenovirus E1A, Ha-RasV12, and MDM2 is sufficient to convert a normal human cell into a cancer cell. Notably, transformation did not require telomerase activation. Therefore, activation of telomere maintenance strategies is not an obligate characteristic of tumorigenic human cells.

Significance

Activation of telomerase, and consequently telomere maintenance, is a common characteristic of human tumors. Existing models of human cancer cells, created by the introduction of defined genetic alterations, all include telomerase activation as an obligate component of the transformed phenotype. Here, we demonstrate that normal human cells can be converted into cancer cells, capable of forming tumors in immunocompromised mice in the absence of telomerase activation or an alternative telomere maintenance strategy. This suggests that alterations in telomere biology must be viewed similarly to genomic instability as catalysts of transformation rather than as central components of the transformed phenotype.

Introduction

Neoplastic transformation occurs via a series of genetic and epigenetic alterations that yield a cell population that is capable of proliferating independently of both external and internal signals that normally restrain growth. For example, transformed cells show reduced requirements for extracellular growth promoting factors, are not restricted by cell-cell contact, and are often immortal (Paulovich et al., 1997; Hanahan and Weinberg, 2000). Through extensive studies of transformation processes in rodent models, it is known that tumor formation can be achieved by the activation of oncogenes and the inactivation of tumor suppressor pathways (Paulovich et al., 1997; Hanahan and Weinberg, 2000; Sherr, 1996). It has long been established that primary rodent cells can be transformed by two oncogenic "hits" such as the combination of ectopic c-myc expression and constitutive activation of Harvey Ras (Ha-RasV12) (Land et al., 1983; Ruley, 1983). However, primary human cells have proven to be refractory to transformation by numerous combinations of cellular and viral oncoproteins, indicative of fundamental differences in requirements for transformation in human versus rodent cells (Sager, 1991; O'Brien et al., 1986; Stevenson and Volsky, 1986; Serrano et al., 1997).

Two major hypotheses have emerged as the underlying explanation for such differences. Primary human and murine cells respond to oncogene activation via homeostatic mechanisms that are proposed to enforce tumor suppression. For example, activation of oncogenes such as c-myc or adenovirus E1A sensitizes primary cells to apoptosis (Debbas and White, 1993; Lowe et al., 1994; Lowe and Ruley, 1993; Harrington et al., 1994; Hermeking and Eick, 1994). Hyper-activation of the ras oncogene or flux through the ras signaling pathway induces a state of terminal growth arrest which is phenotypically similar to cellular senescence (Serrano et al., 1997). In murine cells, the latter response can be bypassed by genetic alterations, which impair the p53 response. Indeed, cells lacking p53 or p19^{ARF} can be transformed directly by activated ras (Kamijo et al., 1997; Serrano et al., 1996, Serrano et al., 1997). In contrast, inactivation of the p53 pathway alone is insufficient to rescue human cells from

4

ras-induced growth arrest (Serrano et al., 1997), suggesting that homeostatic responses in humans flow through multiple independent and redundant effector pathways.

A second characteristic that distinguishes primary human and murine cells is that the latter are easily immortalized (Blasco et al., 1997). Primary human cells rarely undergo spontaneous immortalization, indicating that the control of cellular lifespan is drastically different between these two cell types (Imam et al., 1997; Chin et al., 1999). This phenomenon can be partially attributed to telomere biology. Unlike the embryonic rodent fibroblasts which have served as common models for studies of transformation *in vitro*, primary human fibroblasts have relatively short telomeres and lack detectable telomerase activity (reviewed in McEachern et al., 2000).

The importance of telomerase in human tumorigenesis is supported by numerous observations. First, the majority of human tumors are telomerase-positive (Kim et al., 1994). Second, telomerase activation is sufficient to immortalize some primary human cells in culture (Bodnar et al., 1998; Counter et al., 1998; Wang et al., 1998). Third, telomerase is regulated by an oncogene, c-myc, which is activated in a high percentage of human cancers (Wang et al., 1998).

Previous reports have indicated that primary human fibroblasts and epithelial cells can be transformed by a defined combination of genetic elements, comprising the telomerase catalytic subunit, hTERT, the SV40 early region, and Ha-RasV12 (Hahn et al., 1999; Elenbaas et al., 2001,Hahn et al., 2002). Here we report an alternative model of human cell transformation. We show that co-expression of two oncogenes, adenovirus E1A and Ha-RasV12 is sufficient to enable primary human fibroblasts to grow in an anchorage-independent manner, a hallmark of *in vitro* transformation. However, this combination is insufficient to promote tumor formation in nude mice. Addition of a third oncogene, MDM2, can convert these fibroblasts into cells capable of forming tumors *in vivo*. Interestingly, both anchorage-independent growth *in vitro* and tumorigenesis *in vivo* occur in the absence of telomerase activation. Our results indicate that while telomerase activation is a common characteristic of human tumors, it is not an obligate element of the tumorigenic phenotype.

5

Results

Co-expression of E1A and Ha-RasV12 permits anchorage-independent growth

A defining characteristic of the transformed phenotype is a degree of independence from exogenous mitogenic signals. Many of these signals activate the ras pathway, and activating mutations of *ras* oncogenes or their upstream regulators often occur in human cancers (Barbacid, 1987; Webb et al., 1998). However, in both primary rodent and human cells, expression of the *ras* oncogene alone results in an irreversible growth arrest that is phenotypically similar to cellular senescence (Serrano et al., 1997; Lin et al., 1998). In rodent models, *c-myc* is capable of both bypassing ras-induced growth arrest and cooperating with activated *Ha-RasV12* to transform primary cells into tumorigenic cells (Land et al., 1983). However, combined expression of myc and activated ras in normal human cells not only fails to result in transformation, but also leads to an accelerated appearance of the senescent-like phenotype (data not shown).

Whereas numerous genetic alterations have been shown to bypass ras-induced growth arrest in murine cells, only very few have been shown to be capable of overriding this response in normal human cells. One of these is the ectopic expression of the adenovirus oncogene, E1A (Fig. 1A) (Serrano et al., 1997; de Stanchina et al., 1998). In fact, co-expression of *E1A* and *Ha-RasV12* provided one of the first demonstrations of transformation by cooperating oncogenes in primary rodent cells (Ruley, 1983). Therefore, we tested whether combined expression of E1A and Ha-RasV12 could transform normal human fibroblasts.

One characteristic feature of transformed cells is their ability to grow in the absence of anchorage and, therefore, form colonies in semisolid media. Early passage BJ fibroblasts (normal human foreskin fibroblasts) expressing E1A or Ha-RasV12 individually failed to form colonies in soft agar. In contrast, cells expressing both E1A and Ha-RasV12 were able to form colonies in soft agar with an efficiency comparable to that seen with transformed human and rodent cells (Fig. 1B). For human 293T cells, virtually all plated cells gave rise to colonies compared to a range of 10-30% for BJ/E1A/Ha-RasV12 (for example, see Fig. 1C). In general, colonies generated by BJ/ER (E=E1A, R=Ha-RasV12) contain significantly fewer cells than those generated by 293T cells within the same time period.

6

The role of E1A in the transformation of primary human fibroblasts

E1A is a multifunctional protein that interacts with numerous cellular proteins involved in controlling proliferation. For example, E1A can bind members of the Rb family through conserved motifs designated CR1 and CR2 (Whyte et al., 1988; Harlow et al., 1986; Whyte et al., 1989). Through these interactions, E1A is able to modulate the activity of the E2F family of transcription factors, thus controlling genes required for entry into S phase (Wang et al., 1995; Paulovich et al., 1997; Sherr, 1996). The amino-terminus of E1A binds promiscuous transcriptional co-activators, including p300 (Dorsman et al., 1995; Wang et al., 1995; Goodman and Smolik, 2000). The amino-terminus also binds to the protein complex containing p400, a SWI2/SNF2 family member, and the c-Myc/pCAF-interacting protein, TRRAP (McMahon et al., 1998; Barbeau et al., 1994, Fuchs et al., 2001). This p400 binding region has been shown to be vital for E1A-mediated transformation in mouse cells (Fuchs et al., 2001). The carboxy-terminal region of E1A binds CtBP, a cellular protein, which has been proposed to recruit histone de-acetylases (Goodman and Smolik, 2000).

In order to map the regions and interactions of E1A that are essential for its ability cooperate with Ha-RasV12 in conferring anchorage independent growth to human primary fibroblasts, we used a series of well-characterized deletion mutants for *in vitro* transformation assays (Samuelson and Lowe, 1997). Cells were co-infected with Ha-RasV12 and mutant E1A oncoproteins. While a truncated E1A protein consisting of only the amino-terminal 143 amino acids is unable to bind CtBP (Boyd et al., 1993; Meloni et al., 1999), this mutant is capable of cooperating Ha-RasV12 for colony formation in soft agar with high efficiency (Fig. 1D). Expression of E1A-ΔCR2, a mutant incapable of binding pRb (Samuelson and Lowe, 1997), in combination with Ha-RasV12 invariably led to a senescence-like growth arrest. This result indicated that the interaction between E1A and Rb-family proteins is essential for transformation. Loss of the ability to bind p300 also compromised oncogene cooperation, as did deletion of residues 26-35, indicating that the ability to bind p400 is also critical. Western analysis of E1A mutants suggested that each was expressed similarly to wild-type, with the exception of the CR2 mutant for which the prevalence of cell death in ras co-infected cells made analysis impossible (Figure 1E). Considered together, these results suggest

7

that E1A functions in human cell transformation through concerted effects on multiple cellular pathways that include Rb, p300 and p400.

E1A + Ha-RasV12-expressing cells fail to form tumors

Early passage BJ fibroblasts co-expressing E1A and Ha-RasV12 were tested for the ability to form tumors upon subcutaneous injection into immunocompromised mice. A total of 49 animals were injected in both flanks in a series of five independent experiments. Subject mice were either nude, SCID (beige), or nude mice that had been irradiated as a mean to suppress residual NK (natural killer) responses (Feuer et al., 1995). From a total of 98 injections, only a single tumor formed in a nude, non-irradiated mouse (Figure 1F). This tumor arose after a substantially longer latency (10 weeks) than is normally observed using control cancer cell lines or transformed human 293T cells (~2 weeks), suggesting the possibility that a rare additional genetic alteration may have contributed to tumor formation in this individual case. Thus we conclude that while the combination of E1A and Ha-RasV12 is sufficient to permit anchorage-independent growth of normal human fibroblasts, this combination is insufficient for tumorigenesis in nude mice.

E1A, MDM2, and Ha-RasV12 transform normal human cells into tumor cells

Previous studies of E1A/Ha-RasV12-mediated transformation in primary mouse embryo fibroblasts (MEF) indicated that transformation mediated by this oncogene combination was much more efficient in the absence of p53 (Lowe et al., 1993). In fact, tumors arising from E1A/Ras transformed MEF become apparent only after a long latency period and frequently lack a functional p53 pathway. Interestingly, immunohistochemical analysis of the single tumor produced by the BJ fibroblasts expressing E1A/Ha-RasV12 showed a strong accumulation of nuclear p53; however, results of SSCP analysis excluded the possibility of p53 gene mutations (data not shown).

Accumulation of wild-type p53 is a common feature of human sarcoma, the type of tumor derived from fibroblast precursors. In addition, these tumors often show overexpression of MDM2 (Dei Tos et al., 1997), indicating that negation of p53 function occurs often through mechanisms other than p53 gene mutation. Notably, the tumor that resulted from the E1A/Ha-RasV12-expressing fibroblasts was negative for the

8

expression of p19^{ARF}, an upstream regulator of MDM2 (by immunohistochemistry, data not shown), whereas the pre-injection population of engineered fibroblasts expressed p19^{ARF} abundantly. Guided by these observations, we tested whether negation of the p53 pathway via enforced expression of MDM2 could contribute to the transformation of normal human fibroblasts by E1A and Ha-RasV12.

BJ cells were simultaneously co-infected with three retroviruses that direct the expression of E1A, Ha-RasV12 and MDM2 with each retrovirus bearing a different drug selection marker. Control cells were prepared by replacing individual oncogene-expressing viruses with an empty vector bearing the same selection marker. These triple-infected populations were simultaneously co-selected with puromycin, hygromycin and neomycin for ten days and then either plated into soft agar or injected into immunocompromised mice. Expression of the ectopically expressed oncogenes was confirmed by western blot (not shown). Cell populations expressing E1A/Ha-RasV12/MDM2 formed colonies in soft agar with higher efficiency than BJ/E1A/Ha-RasV12 (see Figs. 1 and 2). Moreover, the triple-infected cells were able to generate tumors when injected subcutaneously into immunocompromised in mice (Fig. 3). Tumors grew to a size at which the animals had to be sacrificed within a period of three to six weeks after injection, a latency comparable to that seen with control human cancer cell lines or with transformed 293T cells (Fig. 3). Tumor formation was observed also when E1A was substituted by the C-terminal deletion mutant E1A-143 (not shown). Histological and immunohistochemical analyses of ERM-derived tumors confirmed the human origin of the tumor cell population and indicated that the neoplasias have features of sarcoma. Moreover immunohistochemistry confirmed the widespread and strong expression of E1A, Ras and MDM2 oncogenes (Fig. 3C)

Cell populations remained polyclonal throughout drug selection *in vitro* and tumorigenesis *in vivo* as revealed by Southern blotting analysis (data not shown). These results argue against the possibility of selection for rare genetic events during tumor formation and support the notion that the combined expression of E1A, MDM2, and Ha-RasV12 is sufficient for the transformation of normal human fibroblasts into tumor cells.

Human fibroblasts transformed by E1A/MDM2/Ha-RasV12 lack telomerase activity

9

Cell immortalization has been posited as a landmark occurrence in the transformation of a normal cell into a cancer cell. Indeed, most human cancers are telomerase-positive, an indirect indication that these cells have acquired a mechanism for both telomere maintenance and extension of proliferative capacity (Kim et al., 1994). In previous reports, transformation of normal human cells absolutely required activation of telomerase via expression of the limiting catalytic subunit, hTERT (Hahn et al., 1999; Elenbaas et al., 2001, Hahn et al., 2002). We previously showed that E1A, Ha-RasV12, and MDM2 were individually incapable of activating telomerase in normal human fibroblasts or epithelial cells (Wang et al., 1998). We therefore tested the possibility that we had transformed normal human cells into cancer cells in the absence of telomerase activation.

Telomerase activity was easily detected in 293T cells using the TRAP assay (Kim et al., 1994; Wright et al., 1995). As few as ten 293T cells were capable of yielding a strong positive signal in our assays. As expected, BJ fibroblasts are telomerase-negative. We similarly fail to detect telomerase activity in BJ cells that have been engineered to express E1A, Ha-RasV12, and MDM2 (BJ/ERM)(Fig. 4A). We conclude that BJ/ERM cells are telomerase-negative, or contain at least 1000-fold less telomerase activity than do 293T cells at the time they are injected into immunocompromised mice.

It is interesting to note that BJ/ERM cells, although able to form colonies in soft agar and tumors in nude mice, are not immortal and, if maintained in culture for an extended period of time (40-50 generations) undergo a "crisis phase" characterized by dramatically reduced proliferation and adoption of a senescent phenotype. Few BJ/ERM cells eventually survive this phase, and these cells become telomerase positive (Fig. 4A, ERM p.c.). This behavior is suggestive of a "telomere crisis" as a consequence of the absence of a telomere maintenance program. This hypothesis is supported by an examination telomere dynamics in BJ/ERM cells. Telomeres shrink continuously as cells are passaged in culture, reaching an average length of ~3 Kb prior to entering a crisis phase from which the population emerges with detectable telomerase activity (Figures 4,5).

Tumors derived from E1A/MDM2/Ha-RasV12 lack telomerase activity

Since ERM-engineered fibroblasts were telomerase-negative at the time of injection into mice, we were curious to ascertain the telomerase status of resultant tumors and to determine whether telomerase activation was a requirement for tumorigenesis. Telomerase activity was measured utilizing the standard TRAP assay described above on tissue sections obtained from the ERM tumors. Whereas a tissue sample from a human tumor produced a robust signal indicative of telomerase activity in a TRAP assay, the lysate from ERM tumor tissue was telomerase-negative (Fig 4B). In order to verify that this negative result was not due to the presence of an inhibitory component within the tissue lysate, we performed a mixing experiment with lysate from 293T cells. When the 293T cell and ERM tumor lysates were mixed in a TRAP reaction, the result was positive, indicating that there was no inhibitory component within the tumor lysate, and thus the tumors were below detectable limits for telomerase activity (Fig 4B).

To verify the forgoing result, we used an independent experimental strategy. In human cells, and in particular in human fibroblasts such as BJ, telomerase activity correlates with the expression of the telomerase catalytic subunit, hTERT (Meyerson et al., 1997; Bodnar et al., 1998). We used an RT-PCR strategy to search for hTERT expression in BJ/ERM tumor specimens. Expression was tested using two independent primer pairs that were chosen for their ability to specifically amplify human TERT without amplifying mouse TERT that might be present from contaminating murine cells in the tumor sample. β-actin mRNA served as an internal control. Ethidium bromide gel staining showed that hTERT mRNA was easily detectable in RNA derived from human cancer cell lines, but BJ/ERM tumors were negative (Figure 4C). To increase the sensitivity of our assay we performed southern blot of the PCR reactions. After southern analysis, one out of 6 tumors analyzed, sample 1659sn, showed weak hTERT expression. This signal was detectable only with an exposure at which the signal of the positive control cells had reached saturation. Considered together, these data suggest that BJ/ERM cells were competent for tumor formation in the absence of telomerase activity and that activation of telomerase can occur late during tumor progression.

Upon explantation into culture BJ/ERM tumor cells, similar to late passage ERM, undergo a crisis event, which is marked by cellular senescence and apparent cell death. In contrast, explantation of tumors generated with 293T control cells did not produce a

11

similar outcome. Instead these cells proliferate robustly. Following this crisis event, few BJ/ERM tumor cells emerge to form a sustainable population. In contrast to early passage BJ/ERM cells and to BJ/ERM tumor samples, and similar to post-crisis late passage BJ/ERM, surviving tumor cells have become telomerase positive (Figure 5A). The forgoing is suggestive of a "telomere crisis" possibly related to the lack of a telomere maintenance program in the tumor mass, a crisis that could be compensated by an *in vitro* selection of a cell population with activated telomerase.

In accord with this hypothesis, TRF assays and telomeric FISH confirmed that continuous telomere erosion occurred during transformation *in vitro* and tumor formation *in vivo*. Telomeres in early passage BJ cells averaged ~7 Kb in length. These became depleted as BJ cells were engineered to express oncogenes and were passaged *in vitro* to an average of 5 Kb at passage 20 and 3.1 Kb in BJ/ERM after the crisis occurred *in vitro*. Consistent with an apparent lack of a telomere maintenance strategy, telomere depletion continued during tumor formation *in vivo* such that explanted cell cultures had extremely short telomeres, averaging 1.6 Kb with 18% of chromosome ends lacking detectable telomeric DNA (Figure 5C,D). These results rule out the possibility that BJ/ERM tumors have activated the recombination-based pathways of telomere maintenance (ALT) (Dunham et al., 2000; Bryan and Reddel, 1997; Hoare et al., 2001; Bryan et al., 1997). Interestingly, when cells explanted from ERM tumors, which had become telomerase-positive *in vitro*, were injected into a second nude mouse, the resultant tumors formed at rates similar to those of primary ERM tumors (data not shown), indicating that telomerase status did not affect the tumorigenicity of ERM cells.

The karyotypes of explanted BJ/ERM cells reveal chromosomal abnormalities characteristic of telomere depletion

As noted above, BJ cells are engineered to express E1A, Ha-RasV12, and MDM2 through simultaneous co-infection. Since these cells have not undergone prolonged expansion in the presence of any individual oncogene in culture, it is not surprising to find that the karyotypes of the engineered cells are normal prior to injection into mice (Figure 6A). Examination of cells that are explanted into culture following tumor formation, however, reveals numerous chromosomal abnormalities (Figures 6B and 6C). In virtually every metaphase, we noted the presence of dicentric chromosomes

12

lacking telomeres at the fusion point that apparently formed via end-to-end fusion of TTAGGG-depleted telomeres. In some metaphases, we also find ring chromosomes (Figure 6B). In addition, these cells showed a very marked aneuploidy as indicated by aberrant number of chromosomes in more than 50% of the metaphases analyzed, also in agreement with aberrant mitosis as a consequence of severe telomeric dysfunction (Figure 6C). These types of genetic abnormalities are a characteristic outcome of telomere depletion and are similar to those seen in the karyotypes of $Terc^{-/-}$ mice (Blasco et al., 1997b; Nanda et al., 1995). Considered together, the results of telomerase detection assays, telomere restriction fragment analyses and cytogenetic examination of explanted tumor cells strongly suggest that combined expression of E1A, Ha-RasV12, and MDM2 is capable of transforming normal human cells into human tumor cells in the absence of direct telomerase activation or alternative mechanisms of telomere maintenance.

Multiple human primary fibroblasts can be transformed by coexpression of E1A/MDM2/Ha-RasV12

In order to verify that E1A/MDM2/Ha-RasV12-mediated transformation is not unique to BJ fibroblasts, we assessed the validity of our transformation model in several additional human primary fibroblasts, including HSF43, WI-38, DET551, SF68 as well as in the human primary mesodermal cells HMSC. Upon co-expression of E1A/MDM2/Ha-RasV12, all were capable of anchorage-independent growth in soft agar (not shown). Efficiencies of colony formation and rates of colony growth were similar to those seen with BJ/ERM cells. Furthermore, these triple-infected fibroblasts were capable of tumor formation when injected into immunocompromised mice (Figure 7A and B)

13

Discussion

Primary rodent cells and animal models have made invaluable contributions to our understanding of neoplastic transformation and of the biology of oncogenes and tumor suppressors. However, it is clear that these models do not perfectly recapitulate the process of tumor development in humans. An early indication of this fact was the inability of human cells to become transformed by the same combinations of oncogenes that could easily transform a variety of normal rodent cells. Recently, the ability to elicit transformation via specific genetic manipulations was extended to normal human cells (Hahn et al., 1999; Elenbaas et al., 2001, Hahn et al., 2002). This has created the opportunity for the development of a variety of defined human cancer models to be used for a detailed study of the cellular pathways that are required for the transformation of normal human cells, and ultimately, to an understanding of any differences in requirements for the transformation of human cells versus those of model organisms. Such information could provide critical insights as rationally designed anti-cancer therapies move from successful applications in animal models to use in humans.

Here we report that normal human fibroblasts can be transformed into cancer cells by combined expression of the adenovirus E1A, Ha-RasV12, and MDM2. As in previous models of human cell transformation, we make use of a combination of viral and cellular oncoproteins that act in a trans-dominant fashion to alter cellular physiology and achieve tumorigenic growth. In accord with previous reports, we show that transformation requires negation of both the Rb and p53 tumor suppressor pathways. Through genetic analyses, we have also identified requirements for interaction with p300 and p400. Both of these cellular proteins are also targeted by SV40 large T-antigen, which is a critical element of the transformation model reported by Weinberg and colleagues (Hahn et al., 1999; Elenbaas et al., 2001). However, recent reports suggest that these are not critical functions of large T, at least in the presence of small t antigen (Hahn et al., 2002).

One striking difference between our results and those reported previously is that in our transformation model we find no requirement for telomerase activation to achieve either anchorage-independent growth *in vitro* or tumor formation *in vivo*. In fact, consistent with their lack of telomerase or other telomere maintenance strategies, our *in*

14

vitro engineered tumor cells show continuous erosion of telomeric repeats. This ultimately leads to genetic instability that is typified by our observation of numerous chromosome end-to-end fusions and pronounced aneuploidy in cells explanted from tumor tissue.

The majority of human cancer cells are telomerase-positive (Kim et al., 1994), and this is long been considered a strong indication that the ability to maintain telomeres is an important step in the development of human cancer. However, it is still debated whether the widespread presence telomerase activity in human tumors is a reflection of a selective expansion of a telomerase-positive stem cell or a selection for a mechanism of telomere maintenance during cancer progression.

Our results are consistent with a model in which telomere maintenance is not essential for transformation, *per se*, but instead serves as a catalyst of tumorigenic conversion and tumor progression. Mouse models have shown that alterations in telomere biology may contribute to tumorigenesis in two ways. We, and others, have previously reported that telomere shortening triggers growth arrest and/or apoptosis, as well as chromosomal end-to-end fusions, leading to premature aging phenotypes in the context of the telomerase-deficient mice. These phenotypes can be rescued by telomerase activation (Lee et al., 1998; Herrera et al., 1999; 2000; Samper et al., 2001). Thus, telomere shortening during the presumably prolonged course of natural tumor development can antagonize tumorigenesis. Indeed $Terc^{-/-}$ mice are more resistant to chemical carcinogenesis and to spontaneous tumor development both in a p16/p19ARF and APC^{min} mutant backgrounds (Greenberg et al., 1999; González-Suárez et al., 2000; Rudolph et al, 2001). However, exhausted telomeres can also compromise chromosome integrity. In fact, in a $p53^{+/-}$ background, $Terc^{-/-}$ mice show higher levels of chromosomal instability and a higher incidence of cancer, suggesting that short telomeres actually act as a pro-oncogenic factor under certain conditions (Chin et al., 1999).

Our data suggest that, while changes in telomere biology are undoubtedly important for the course of natural tumor development, telomere maintenance is not required for the creation of human cancer cells by acute alterations in oncogenes and tumor suppressors. Rather, in our human transformation model the activation of

15

telomere maintenance strategies becomes important only during prolonged expansion of tumor cells to restore genomic stability to an extent that permits cell survival.

Using oncoprotein mutants and genetic complementation, we find that inactivation of the Rb and p53 tumor suppressor pathways is critical for this transformation process. Furthermore, we find that the ability of E1A to target p300 and p400 is essential for its ability to function as a human oncogene. It will also be of interest to determine whether MDM2 contributes to the transformation of human cells solely through its ability to antagonize p53 or also via effects on additional cellular pathways.

The war on cancer is predicated on the notion that increased understanding of the biology of cancer cells might reveal an "Achilles heel" that can be exploited as an effective and specific therapeutic target. The use of rodent cell culture and animal models have been the most informative vehicles in the drive toward this goal. However, the availability of defined human cell transformation models will allow us to build toward a complete understanding of the biological pathways that must be altered to achieve tumorigenic conversion of normal cells.

Experimental Procedures

Cells

BJ normal human foreskin fibroblasts were maintained in Minimum Essential Medium with Earle's salts (MEM) supplemented with non-essential amino acids (NEAA) and 10% fetal bovine serum (FBS) (Gibco BRL). The amphotropic packaging cell line, LinX-A (Hannon et al., 1999), 293T, Detroit 551, WI-38, HSF43, SF68 cells were maintained in Dulbecco's Modified Eagle culture medium (DMEM), supplemented with 0.01% Sodium Pyruvate and 10% FBS. HMSC human primary mesodermal cells (Poietics, BioWhittaker) were grown in MSCGM synthetic medium (Poietics, BioWhittaker). All cells were cultured at 37°C in the presence of 5% CO_2.

Retroviral Infection

pBABE-Puro Ha-rasV12, Wzl-Neo E1A 12s, pHygroMaRX mdm2, and corresponding empty retroviral vectors were used to singularly transfect the amphotropic packaging cell line LinX-A. Transfection was performed by the calcium phosphate method. At 72 hours post-transfection, viral supernatants were collected, filtered, supplemented with 4 μg/ml polybrene and combined in order to obtain the oncogene combinations described in the text. In cells where only one or two oncogenes were used to infect the primary cells, corresponding empty vectors replaced the omitted oncogenes so that infected cells were equally resistant to all the selection drugs used (hygromycin, puromycin and neomycin). The proper viral mix was then used to infect early passage human primary fibroblasts (BJ, Detroit 551, WI-38, HSF43, SF68) and human primary mesodermal cells (HMSC). After infection, cells were selected with a combination hygromycin (50 μg/ml), puromycin (1 μg/ml) and neomycin (300 μg/ml) for 7 days. Effective infection was confirmed by western blot analysis.

Western Blot analysis

Western blotting was performed essentially as described by Harlow and Lane (1988). Cells were washed with cold PBS and lysed in Laemmli loading buffer. Lysates were heated at 95°C for 10 min. Samples were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated with the following mouse monoclonal antibodies: E1A-specific M73 and M58 antibody; c-Ha-ras (OP23) (Oncogene Research Products); mdm2-spcific antibody (4B2)

17

(a kind gift from A. Levine); bcl2 (C2) (Santa Cruz); p53 (DO-1) (Santa Cruz). Immune complexes were visualized by secondary incubation with a sheep anti-mouse HRP-conjugated secondary antibody (Amersham). Blots were developed by enhanced chemiluminescence (Amersham).

Anchorage-independent Growth

Human primary fibroblasts uninfected, infected with a control empty virus or expressing different oncogenes (E1A, Ha-rasV12, mdm2, dnp53, bcl2), alone or in combination, were analyzed for anchorage-independent growth in semi-solid media. Approximately 10^5 cells were plated in 0.3% low melting point agarose/growth media onto 60-mm dishes with a 0.5% agarose underlay. Fresh top agar was added weekly. Colonies were photographed after 2 weeks.

Subcutaneous Tumorigenicity Assay

For the tumorigenicity assays, eight-week-old immunocompromised athymic nude mice (Hsd:Athymic nude-nu, Harlan) were used. Cells (5×10^6) were resuspended in 100 µl of PBS and injected with a 25-gauge needle into anaesthetized mice. BJ cells infected with the E1A/ras combination were also injected into 10 nude mice γ-irradiated with 400 rad prior injection and into 6 SCID beige mice (C.B-17/lcrHsd-scid-bg, Harlan). Tumor size was monitored every 5 days. Mice were sacrificed when the tumors reached a diameter of 1-1.2 cm or after 16 weeks of monitoring.

Tumors were collected in a sterile field and minced. Tumor fragments were immediately frozen in liquid nitrogen for DNA and protein extraction and for telomerase assays. Other fragments were fixed in 10% formalin for histological and immunohistochemical examinations. Finally, fragments were finely minced, washed in PBS and plated in culture medium for isolation of tumor cells.

Tumor morphological and histochemical examination

Formalin fixed/paraffin embedded or snap frozen fragments of tumor specimens were stained with hematoxylin and eosin and with histochemical stains (PAS and PAS after diastase, Reticulum and Masson's trichrome stain) for morphological evaluation and histochemical analyses.

Immunohistochemistry was performed with the peroxidase ARK kit (DAKO Glostrup Denmark) and DAB as chromogen. The following primary antibodies were

utilized: Intermediate filaments (Vimentin, pan-keratin, desmin) and other human specific monoclonal antibodies (S100 protein, EMA, CD45) were used as histogenetic markers. The expression of etopically-expressed oncogenes was determined by using monoclonal antibodies specific for MDM2 (4B2) (a kind gift from A. Levine), p21-ras (OP23) (Oncogene Research Products) and E1A (M73).

Scoring of chromosomal abnormalities

Karyotype analysis

Metaphase chromosomes preparation from explanted tumor cells and quinacrine banding (QFQ staining) were according to standard protocols (Barch et al., 1997)

Q-FISH

The indicated numbers of metaphases from each culture were scored for chromosomal aberrations by superimposing the telomere image on the DAPI chromosome image in the TFL-telo software (gift from Dr. Peter Lansdorp, Vancouver). End-to-end fusions, can be 2 chromosomes fused by their p-arms (Robertsonian-like fusions) or 2 chromosomes fused by their q-arms (dicentrics).

Clonality analysis

To confirm the polyclonality of tumor cell population, genomic DNA was extracted from parental and explanted tumor cells by conventional Proteinase K/SDS digestion. Twelve micrograms of DNA were digested with either BamHI , BamHI plus XhoI or BamHI plus SalI and fractionated in a 0.8% agarose gel. After transfer onto Hybond N+ membrane (Amersham), blots were hybridized with ^{32}P-labeled probes specific for mdm2, E1A or Ha-ras. Membranes were hybridized overnight at 65°C in 0.2 M NaPO$_4$, 1 mM EDTA, 7% SDS, 1% BSA in the presence of 15% formamide. Membranes were washed twice in 0.1% SDS, 0.2X SSC and once in 0.1X SSC at 60°C, followed by autoradiography.

Telomere length measurements

Q-FISH on metaphasic chromosomes

Metaphases were prepared for Q-FISH and hybridized as described (Samper et al., 2000; 2001). To correct for lamp intensity and alignment, images from fluorescent beads (Molecular probes, USA) were analyzed using the TFL-Telo program. Telomere fluorescence values were extrapolated from the telomere fluorescence of LY-R (R cells)

and LY-S (S cells) lymphoma cell lines of known lengths of 80 and 10 Kb (McIlrath et al. 2001). There was a linear correlation (r2=0.999) between the fluorescence intensity of the R and S telomeres with a slope of 38.6. The calibration-corrected telomere fluorescence intensity (ccTFI) was calculated as described (Herrera et al., 1999).

Images were captured using Leica Q-FISH software at 400 mSec integration-time in a linear acquisition mode to prevent over-saturation of fluorescence intensity and recorded using a COHU CCD camera on a Leica Leitz DMRB fluorescence microscope. TFL-Telo software (gift from Dr. Lansdorp, Vancouver), was used to quantify the fluorescence intensity of telomeres from at least 10 metaphases of each data point. The images of metaphases from different cell cultures were captured on the same day, in parallel, and scored blind.

Terminal restriction fragment analysis (TRF)

Cell were prepared in agarose plugs and digested with Mbo I for TRF analysis using pulse-field eletrophoresis as described in Blasco et al. (1997).

Telomerase assays

Telomerase activity was measured with a modified telomeric repeat amplification protocol (TRAP), as described (Blasco et al., 1997).

Analysis of hTERT mRNA expression by RT-PCR

Analysis of human TERT expression was carried out by RT-PCR. cDNA was synthesized from 1 µg of total RNA using random primers in a 20 µl reaction. 1 µl of cDNA was then used to amplify two fragments of human TERT sequence, both spanning an intronic sequence. Primers hTERT1s, 5'-TTCCTGCACTGGCTGATGAGTGT-3' and hTERT1a, 5'-AGCGTCGGCCCTCTTTTCTCTG-3' were used to amplify a 330 bp fragment spanning exons 3 and 4 of the human TERT sequence. Primers hTERT2s, 5'-ACAGCACTTGAAGAGGGTG-3' and hTERT2a, 5'- GTGCCTTCACCCTCGAGG-3' were used to amplify a 210 bp fragment spanning exons 4 and 5 of the human TERT sequence. Both PCR reactions were carried out for 40 cycles with an elongation at 72°C for 30 sec and annealing at 65°C. PCR products were analyzed on a 3% agarose gel. The quality of cDNA was controlled by PCR amplification of a 500 bp fragment of a β-actin transcript in a 20 cycle-PCR reaction.

References

Barbacid, M. (1987). Ras genes. Ann. Rev. Biochem. *56,* 779-827.

Barbeau, D., Charbonneau, R., Whalen, S.G., Bayley, S.T., and Branton, P.E. (1994). Functional interactions within adenovirus E1A protein complexes. Oncogene. *9,* 359-373.

Blasco, M.A., Lee, H.W., Rizen, M., Hanahan, D., DePinho, R., and Greider, C.W. (1997a). Mouse models for the study of telomerase. Ciba Found. Symp. *211,* 160-176.

Blasco, M.A., Lee, H.W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A., and Greider, C.W. (1997b). Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell. *91,* 25-34.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. (1998) Extension of life-span by introduction of telomerase into normal human cells. Science. *279,* 349-352.

Boyd, J.M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S., and Chinnadurai, D. (1993). A region in the C-terminus of adenovirus 2/5 E1A protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis, and metastasis. EMBO J. *12,* 469-478.

Bryan, T.M., and Reddel, R.R. (1997). Telomere dynamics and telomerase activity in *in vitro* immortalised cells. Eur J Cancer. *33,* 767-773.

Bryan, T.M., Englezou, A., Dalla-Pozza, L., Dunham, M.A., and Reddel, R.R. (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat. Med. *3,* 1271-1274.

Chin, L., Artandi, S.E., Shen, Q., Tam, A., Lee, S.L., Gottleib, G.J., Greider, C.W., and DePinho, R.A. (1999). p53-deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. Cell. *97,* 527-538.

Counter, C.M., Hahn, W.C., Wei, W., Caddle, S.D., Beijersbergen, R.L., Lansdorp, P.M., Sedivy, J.M., and Weinberg, R.A. (1998). Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. Proc. Natl. Acad. Sci. USA. *95*, 14723-14728.

Debbas, M. and White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. *7*, 546-554.

Dei Tos, A.P., Piccinin, S., Doglioni, C., Vukosavljevic, T., Mentzel, T., Boiocchi, M., and Fletcher, C.D. (1997). Molecular aberrations of the G1-S checkpoint in myxoid and round cell liposarcoma. Am. J. Pathol. *151*, 1531-1539.

de Stanchina, E., McCurrach, M.E., Zindy, F., Shieh, S.Y., Ferbeyre, G., Samuelson, A.V., Roussel, M.F., Sherr, C.J., and Lowe, S.W. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev. *12*, 2434-2442.

Dorsman, J.C., Hagmeyer, B.M., Veenstra, J., Elfferich, N.N., Zantema, A., and Van Der Eb, A.J. (1995). The N-terminal region of the adenovirus type 5 E1A proteins can repress expression of cellular genes via two distinct but overlapping domains. J. Virol. *69*, 2962-2967.

Dunham, M.A., Neumann, A.A., Fasching, C.L., and Reddel, R.R. (2000). Telomere maintenance by recombinantion in human cells. Nat. Genet. *26*, 447-450.

Elenbaas, B., Spirio, L., Koerner, F., Fleming, M.D., Zimonjic, D.B., Donaher, J.L., Popescu, N.C., Hahn, W.C., and Weinberg, R.A. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. *15*, 50-65.

Feuer, G., Stewart, S.A., Baird, S.M., Lee, F., Feuer, R., and Chen, I.S. (1995). Potential role of natural killer cells in controlling tumorigenesis by human T-cell leukemia viruses. J. Virol. *69*, 1328-1333.

Fuchs, M., Gerber, J., Drapkin, R., Sif, S., Ikura, T., Ogryzko, V., Lane, W.S., Nakatani, Y., and Livingston, D.M. (2001). The p400 complex is an essential E1A transformation target. Cell. *106*, 297-307.

Gonzalez-Suarez, E., Samper, E., Flores, J.M., Blasco, M.A. (2000). Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. Nat Genet. *26*, 114-117.

Goodman, R.H. and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. *14*, 1553-1577.

Greenberg, R.A., Chin, L., Femino, A., Lee,K.H., Gottlieb, G.J., Singer, R.H., Greider, C.W., DePinho,R.A. (1999). Short dysfunctional telomeres impair tumorigenesis in the INK4a(delta2/3) cancer-prone mouse. Cell. *97*, 515-525.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumor cells with defined genetic elements. Nature. *400,* 464-468.

Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., Weinberg, R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol Cell Biol. *22*, 2111-2123.

Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. Cell. *100,* 57-70.

Hannon, G.J., Sun, P., Carnero, A., Xie, L.Y., Maestro, R., Conklin, D.S., and Beach, D. (1999). MaRX: an approach to genetics in mammalian cells. Science. *283*, 1129-1130.

Harlow, E., Whyte, P., Franza, B.R., Jr., and Schley, C. (1986). Association of adenovirus early-region 1A proteins with cellular polypeptides. Mol Cell Biol. *6,* 1579-1589.

Harrington, E.A., Bennett, M.R., Fanindi, A., and Evan, G.I. (1994). c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J. *13*, 3286-3295.

23

Hermeking, H. and Eick, D. (1994). Mediation of c-Myc-induced apoptosis by p53. Science. *265*, 2091-2093.

Herrera, E., Samper, E., Martin-Caballero, J., Flores, J.M., Lee, H.W., and Blasco, M.A. (1999). Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. EMBO J. *18*, 2950-2960.

Herrera, E., Martinez-A, C., and Blasco, M.A. (2000). Impaired germinal center reaction in mice with short telomeres. EMBO J. *19*, 472-81.

Hoare, S.F., Bryce, L.A., Wisman, G.B., Burns, S., Going, J.J., van der Zee, A.G., and Keith, W.N. (2001) Lack of telomerase RNA gene hTERC expression in alternative lengthening of telomeres cells is associated with methylation of the hTERC promoter. Cancer Res. *61*, 27-32.

Imam, S.A., Kim, M.S., Anker, L., Datar, R.H., Law, R.E., and Taylor, C.R. (1997). Systematic determination of telomerase activity and telomere length during the progression of human breast cancer in cell culture models. Anticancer Res. *17*, 4435-4441.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science. *266,* 2011-2015.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature. *304,* 596-602.

Lee, H.W., Blasco, M.A., Gottliebb, G.J., Horner, J.W. 2nd, Greider, C.W., and DePinho, R.A. (1998). Essential role of mouse telomerase in highly proliferative organs. Nature. *392,* 569-574.

24

Lin, A.W., Barradas, M., Stone, J.C., van Aelst, L. Serrano, M., and Lowe, S.W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev. *12,* 3008-3019.

Lowe, S.W., and Ruley, H.E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. *7,* 535-545.

Lowe, S.W., Bodis, S., McClatchey, A., Remington, L., Ruley, H.E., Fisher, D.E., Housman, D.E., and Jacks, T. (1994). p53 status and the efficacy of cancer therapy in vivo. Science. *266,* 807-810.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G., and Sherr, C.J. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell. *91,* 649-659.

McEachern, M.J., Krauskopf, A., and Blackburn, E.H. (2000). Telomeres and their control. Ann. Rev. Genet. *34,* 331-358.

Meloni, A.R., Smith, E.J., and Nevins, J.R. (1999). A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor. Proc. Natl. Acad. Sci. USA. *96,* 9574-9579.

Meyerson, M., Counter, C.M., Eaton, E.N., Ellisen, L.W., Steiner, P., Caddle, S.D., Ziaugra, L., Beijersbergen, R.L., Davidoff, M.J., Liu, Q., Bacchetti, S., Haber, D.A., and Weinberg, R.A. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells during immortalization. Cell *90,* 785-95.

Nanda, I., Schneider-Rasp, S., Winking, H., and Schmid, M. (1995). Loss of telomeric sites in the chromosomes of mus musculus domesticus (Rodentia: Muridae) during Robertsonian rearrangements. Chromosome Res *3,* 399-409.

O'Brien, W., Stenman, G., and Sager, R. (1986). Suppression of tumor growth by senescence in virally transformed human fibroblasts. Proc. Natl. Acad. Sci. USA *83,* 8659-8663.

Paulovich, A.G., Toczyski, D.P., Hartwell, L.H. (1997). When checkpoints fail. Cell. *88,* 315-322.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature. *304,* 602-606.

Sager, R. (1991). Senescence as a mode of tumor suppression. Environ. Health Perspect. *93,* 59-62.

Samper, E., Goytisolo, F.A., Menissier-de Murcia, J., Gonzalez-Suarez, E., Cigudosa, J.C., de Murcia, G., and Blasco, M.A. (2001). Normal telomere length and chromosomal end capping in poly (ADP-ribose) polymerase-deficient mice and primary cells despite increased chromosomal instability. J Cell Biol. *154,* 49-60.

Samuelson, A.V. and Lowe, S.W. (1997). Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins. Proc. Natl. Acad. Sci. USA. *94,* 12094-12099.

Sherr, C.J. (1996). Cancer cell cycles. Science. *274,* 1672-1677.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D.H., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell. *88,* 593-602.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R.A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell. *85,* 27-37.

Stevenson, M. and Volsky, D.J. (1986). Activated v-myc and v-ras oncogenes do not transform normal human lymphocytes. Mol. Cell. Biol. *6,* 3410-3417.

Wang, H.H., Moran, E., and Yaciuk, P. (1995). E1A promotes association between p300 and pRb in multimeric complexes required for normal biological activity. J. Virol. *69,* 7917-7924.

Wang, J., Xie, L.Y., Allan, S., Beach, D., and Hannon, G.J. (1998). Myc activates telomerase. Genes Dev. *12*, 1769-1774.

Webb, C.P., Van Aelst, L., Wigler, M.H., and Woude, G.F. (1998). Signaling pathways in Ras-mediated tumorigenicity and metastasis. Proc. Natl. Acad. Sci. USA. *95*, 8773-8778.

Whyte, P., Buchkovich, K.J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature. *334,* 124-129.

Whyte, P., Williamson, N.M. and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. Cell. *56,* 67-75.

Wright, W.E., Shay, J.W., and Piatyszek, M.A. (1995). Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity, and sensitivity. Nucleic Acids Res. *23,* 3794-3795.

Acknowledgements

Figure Legends
Figure 1.
Transformation of normal human fibroblasts by E1A and Ha-RasV12

A. Normal human diploid fibroblasts (BJ) infected with a recombinant retrovirus that directs expression of Ha-RasV12 stain positively for expression of the senescence-associated β-galactosidase marker whereas BJ fibroblasts co-infected with retroviruses for E1A (12s) and Ha-RasV12 expression do not stain for this marker and continue to proliferate.

B. BJ fibroblasts were infected with retroviruses for E1A or Ha-RasV12 expression alone or in combination and assayed for colony formation in semi-solid media.

C. Colonies containing greater than 100 cells were counted from triplicate platings of cells with the indicated genotype to determine the rate of colony formation. Standard error from the mean is indicated.

D. A series of well-characterized functional mutants of E1A were co-expressed in BJ fibroblasts with Ha-RasV12 and assayed for the ability to form colonies in semi-solid media. Only the E1A-143 mutant, which is defective for the ability to bind CtBP, was capable of forming colonies in this assay, indicating that the ability of E1A to bind p300, p400, and Rb is critical for E1A-mediated transformation.

28

E. Western blot analysis indicated that the E1A functional mutants were well-expressed as compared to wild-type, and thus the defects in colony formation were not due to E1A not being adequately expressed.

F. BJ fibroblasts co-expressing E1A and Ha-RasV12 were unable to result in tumor formation in nude mice. There was one instance in which a single tumor formed in the flank of a bilaterally injected mouse, which is denoted as 1*. This tumor arose after a much greater latency period, indicating the possibility for the selection of a mutation *in vivo*.

Figure 2.

MDM2 cooperates with E1A and Ha-RasV12 to promote colony formation *in vitro*

A. BJ fibroblasts were infected with retroviruses to direct the expression of three genes, E1A (E), Ha-RasV12 (R), and MDM2 (M), alone and in combination and plated into semi-solid media. Notably, cells engineered to express all three genes simultaneously (ERM) formed robust colonies similar to those produced in the 293T control.

B. Colonies containing greater than 100 cells were counted from triplicate platings of cells with the indicated genotype in order to determine the rate of colony formation. The standard error from the mean is indicated. ERM cells form colonies at a rate greater than ER cells and more comparable to the control 293T cell line.

Figure 3.

Conversion of BJ fibroblasts into tumor cells by combined expression of E1A, Ha-RasV12 and MDM2

A. Examples of immunocompromised mice (nude, no γ-irradiation) that have been injected with either control BJ fibroblasts or BJ cells that have been engineered to express E1A (E), Ha-RasV12 (R), and MDM2 (M).

B. Tumor growth rates from two representative mice injected in both flanks with BJ/ERM fibroblasts are compared to tumor growth rates in a mouse that had been injected in both flanks with E1A-expressing 293T cells, as indicated.

C. Immunohistochemistry staining of ERM tumor samples with antibodies to E1A, Ha-Ras, and MDM2, verifying that the tumors were in fact derived from the injected cells.

D. A tabulation of tumor formation by BJ/ERM cells in nude mice.

Figure 4.

BJ/ERM cells are telomerase-negative upon injection into nude mice

A. The TRAP assay was used to detect telomerase activity in uninfected BJ cells and BJ/ERM cells at various passages. At the time of injection (p20), ERM cells are telomerase negative. However, after continuous passaging in culture, these cells undergo an event similar to crisis, a point at which many cells undergo apoptosis or senescence. Cells that emerge from this crisis event become telomerase positive (ERM p.c.). The indicated protein concentrations (µg) of S-100 extract were used. The highest protein concentration, 2.3 µg, was also pre-treated with RNase (R) as a negative control.

B. Tumors were recovered from mice injected with BJ/ERM cells and assayed for the presence of telomerase activity using the TRAP assay. To test whether tissue extracts contained inhibitors of any step of the procedure, we mixed lysate derived from 1,000 telomerase-positive 293T cells with the tumor extract. This produced a positive signal. For comparison, a similar telomerase assay was performed using a mass-equivalent portion of lysate from a human breast tumor is shown.

C. RT-PCR was used to detect hTERT expression in tumor samples. Expression was tested by using two primer primer pairs that would allow for the specific amplification of hTERT and not mouse TERT that might be present from contaminating murine cells within the tissue sample. β-actin served as an internal control. Sensitivity of the assay was increased by performing a Southern blot of the PCR reactions for hTERT.

Figure 5.

Telomere dynamics in ERM transformed cells before and after tumor explant

A. The TRAP assay was used to detect telomerase activity in cells explanted from ERM tumors and compared to ERM cells that had been in continuous culture. The explanted cell lines (1662dxp8, 1662snp18, and 1731snp9) were telomerase positive, similar to what was seen in ERM cells that had been continuously passaged in culture until they underwent crisis (ERM p.c.).

B. Telomere length in uninfected BJ, BJ/ERM, and ERM tumor explants were analysed by TRF using pulse-field electrophoresis. As predicted, the telomeres of uninfected BJ and BJ/ERM become shorter as the cells are passaged *in vitro.* Interestingly, the telomeres of both ERM p.c. and explanted cell lines (1662dxp8, 1662snp18, and 1731snp9) had become extremely short irrespective of the fact they were telomerase-positive.

C. Telomere length distribution of BJ, BJ/ERM and BJ/ERM tumor cells at different passages as determined by quantitative FISH (Q-FISH) using a telomeric PNA probe. Average telomere length in kbps and standard deviation, as well as, the total number of telomeres analyzed for each cell culture are indicated. The percentage of undetectable telomeres using Q-FISH is also indicated.

D. Representative Q-FISH images of metaphases from BJ, BJ/ERM and BJ/ERM tumor cells hybridized with a telomeric PNA probe. We note a significant decrease in telomeric signal, as well as the increased chromosomal instability and increased aneuploidy in later passage EMR cells and in the tumor cell line. Blue: DAPI; Yellow dots: telomeres.

Figure 6.

Karyotypes of ERM cells post-explantation indicate chromosomal abnormalities characteristic of telomere depletion

A. Karyotypic analysis of normal BJ fibroblasts and BJ/ERM cells by G-banding is shown. No cytogenetic abnormalities were detected in the majority of metaphases.

B. Representative metaphases from BJ/ERM cells explanted into culture following tumor formation are shown. In virtually every metaphase, we note one or more chromosomal abnormalities, including end-to-end fusions and ring chromosomes. This is correlated with a high degree of cell death during the explantation procedure and may reflect this cell population entering a crisis phase during telomere depletion.

C. Quantification and representative image of cytogenetic alterations detected in metaphases from different passage BJ, BJ/ERM and BJ/ERM tumor cells after hybridization with DAPI and a fluorescent Cy-3-labeled telomeric peptide nucleic acid (PNA) probe. Blue, chromosome DNA stained with DAPI; yellow and white dots, TTAGGG repeats. Karyotype analysis is BJ, BJ/ERM and BJ/ERM tumor

31

cells at different passages is also shown. For each culture, 15 metaphases were evaluated. Notice that the tumor-derived cell lines were severely aneupliod compared with BJ cells at two different passages. ERM p20 and ERM pc cells showed a modest aneuploidy. FRAGM: chromosome fragment; DICENT: dicentric; TRICENT: tricentric; TRANSL: translocation.

Figure 7.
**Multiple human fibroblast cell lines can be transformed by the combined expression of E1A, Ha-RasV12, and MDM2**

A. Examples of immunocompromised mice (nude, no γ-irradiation) injected with control fibroblasts expressing E1A + Ha-RasV12 or fibroblasts engineered to express the oncogene combination of E1A + Ha-RasV12 + MDM2. As had been seen with BJ fibroblasts, additional human skin fibroblasts (HSF43, WI-38, and Detroit551) became tumorigeneic upon expression of the combination of ERM, while the combination of ER alone was unable to result in tumorigenesis.

B. Table quantifying the frequency of tumor formation by different human fibroblast lines expressing ERM.
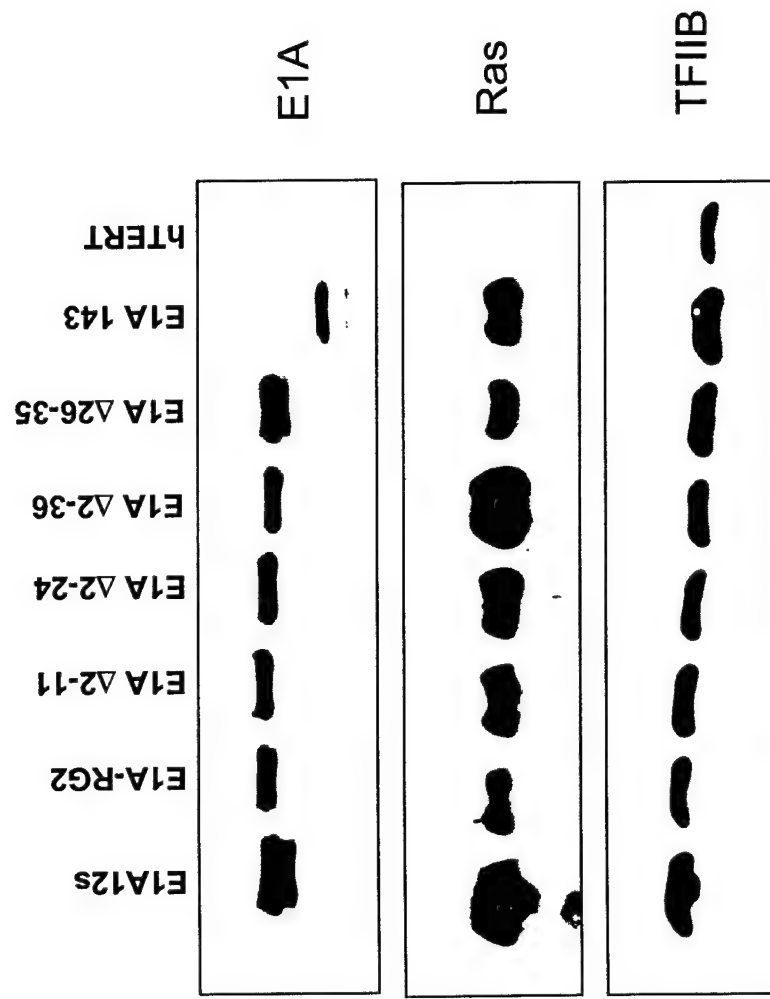
Seger et al., Figure 1A, 1B, and 1C

A.

B.

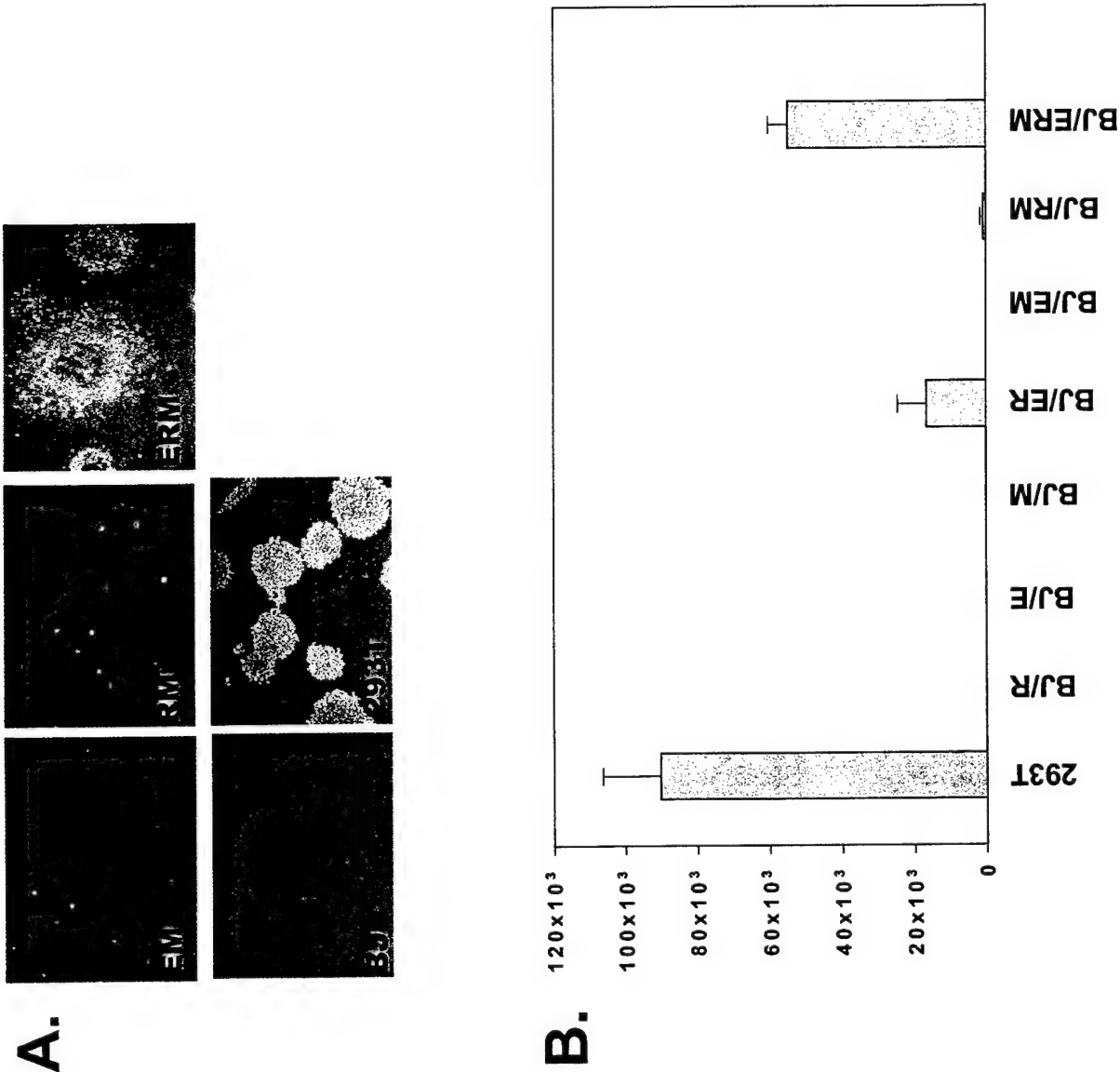**C.**

Seger et al., Figure 1D

D.

Seger et al., Figure 1E

E.

Seger et al., Figure 1F
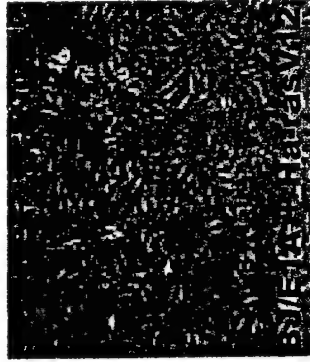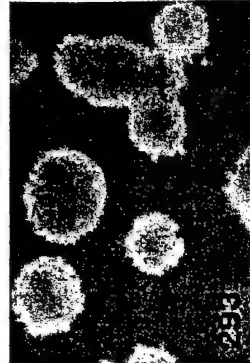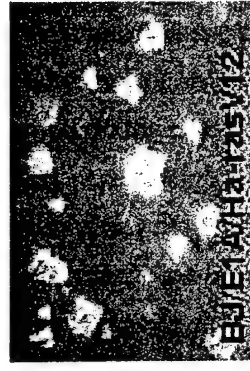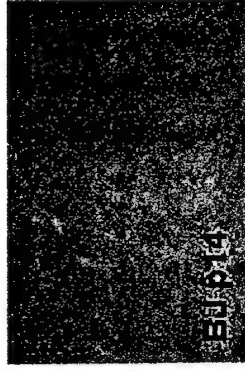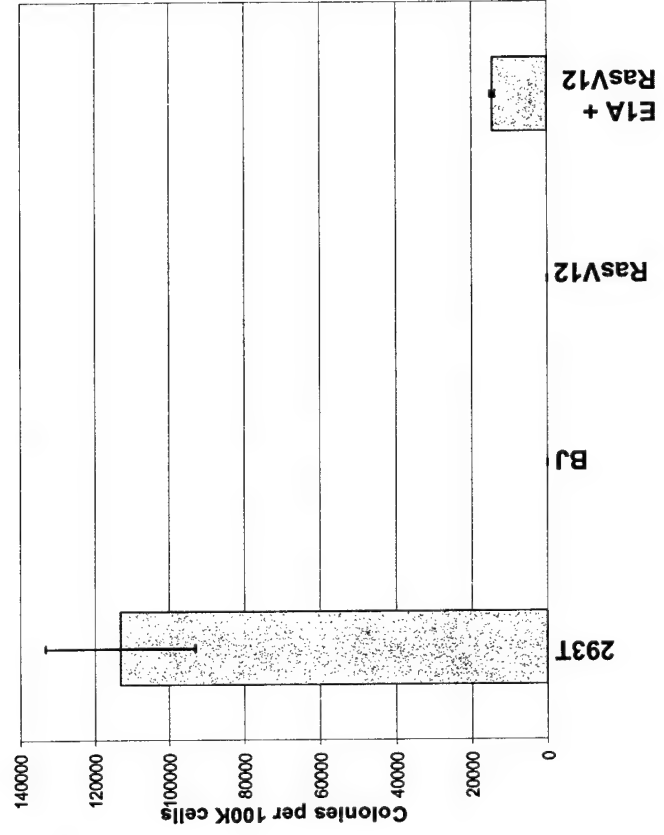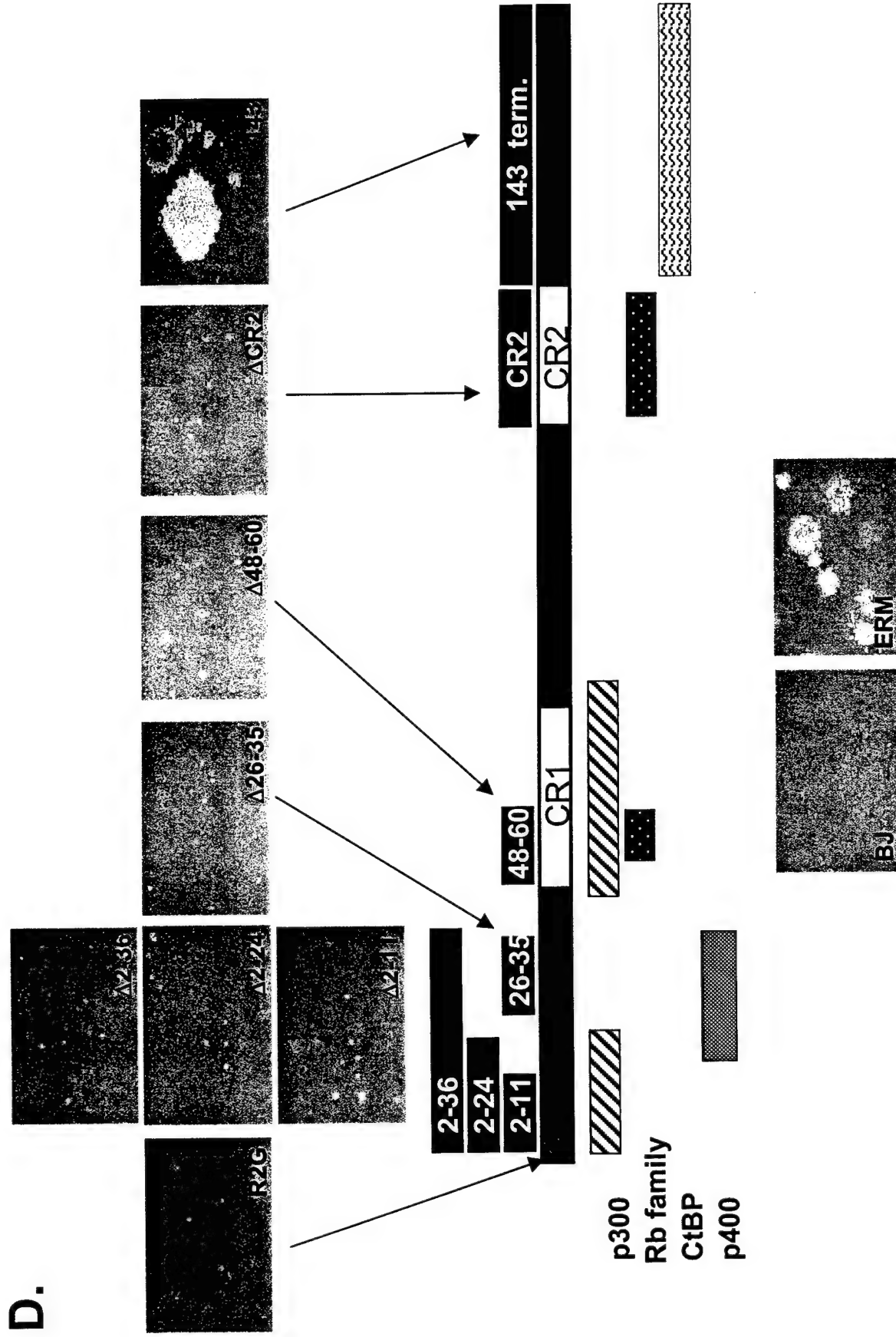
F.

Tumor Formation in Immunodeficient Mice: E1A + Ha-RasV12

Cell Line	Number Tumors/Number Injections
Non-Irradiated Nudes:	
293T	8/8
BJ P18	0/10
BJ/E1A + Ha-RasV12 (serial)	1*/8
BJ/E1A + Ha-RasV12 (coinfection)	0/10
Irradiated Nudes:	
293T	2/2
BJ P18	0/2
BJ/E1A + Ha-RasV12 (coinfection)	0/4
SCID-beige:	
293T	2/2
BJ P18	0/2
BJ/E1A + Ha-RasV12 (serial)	0/4
BJ/E1A + Ha-RasV12 (coinfection)	0/6

Seger et al., Figure 2A and 2B
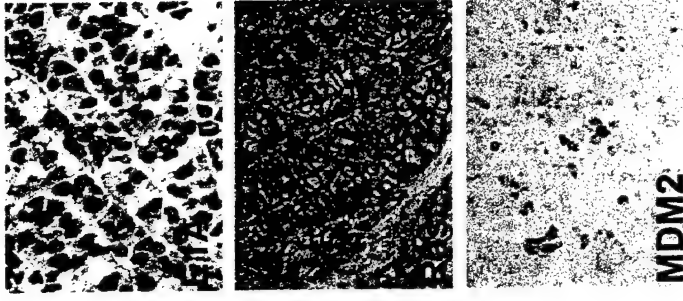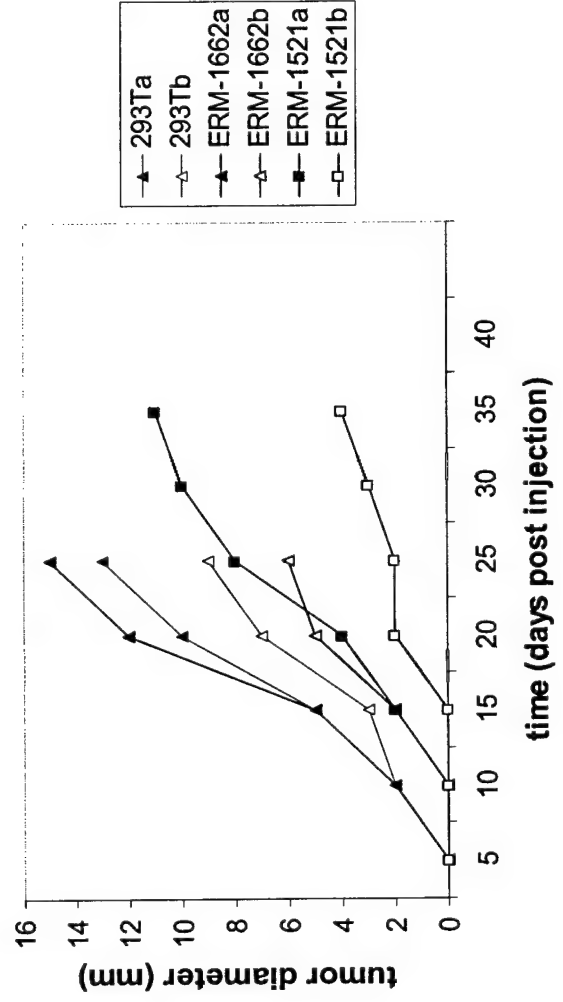
Seger et al., Figure 3A and 3B

A.

BJ

BJ/ERM

B.

Tumor growth rates

tumor diameter (mm)

time (days post injection)

- 293Ta
- 293Tb
- ERM-1662a
- ERM-1662b
- ERM-1521a
- ERM-1521b

C.

MDM2

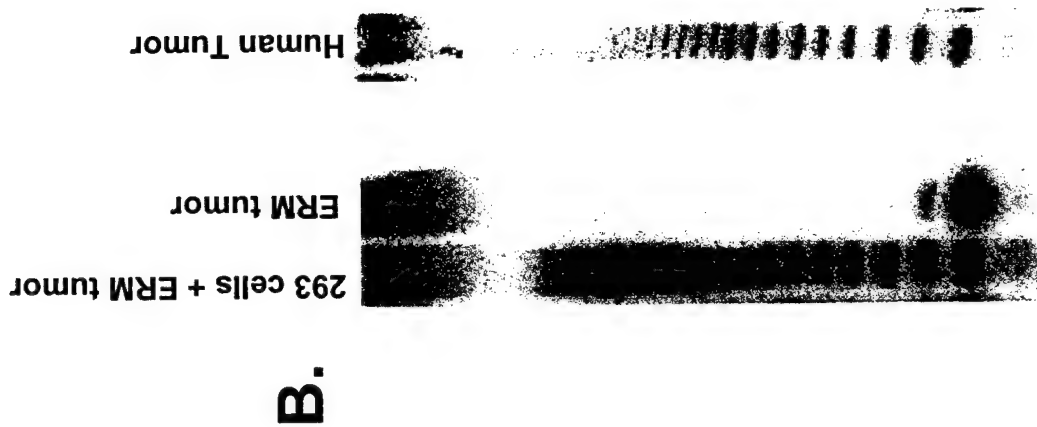Seger et al., Figure 3D

D.

Tumor Formation in Immunodeficient Mice: E1A + MDM2 + Ha-RasV12

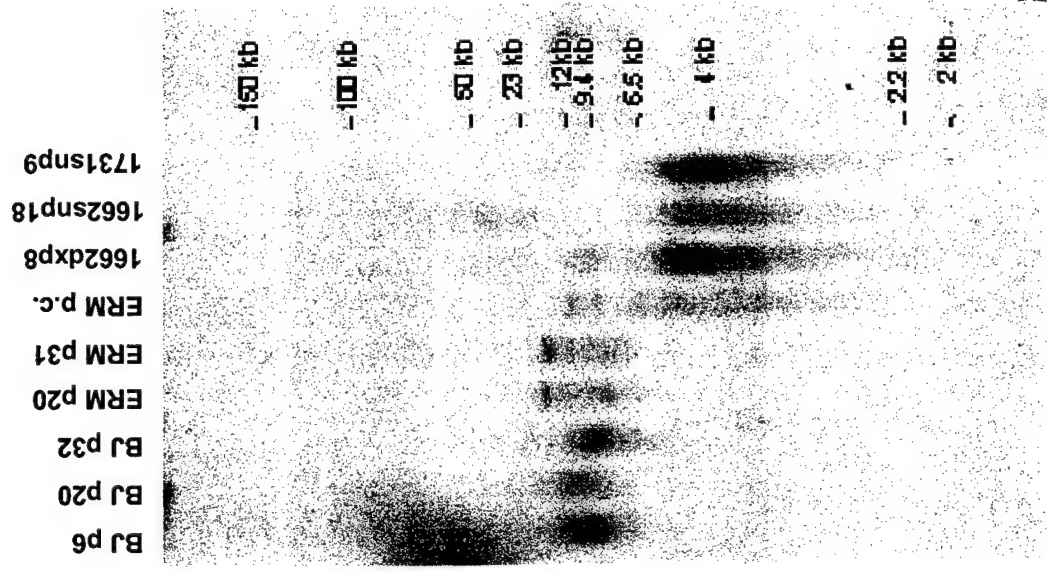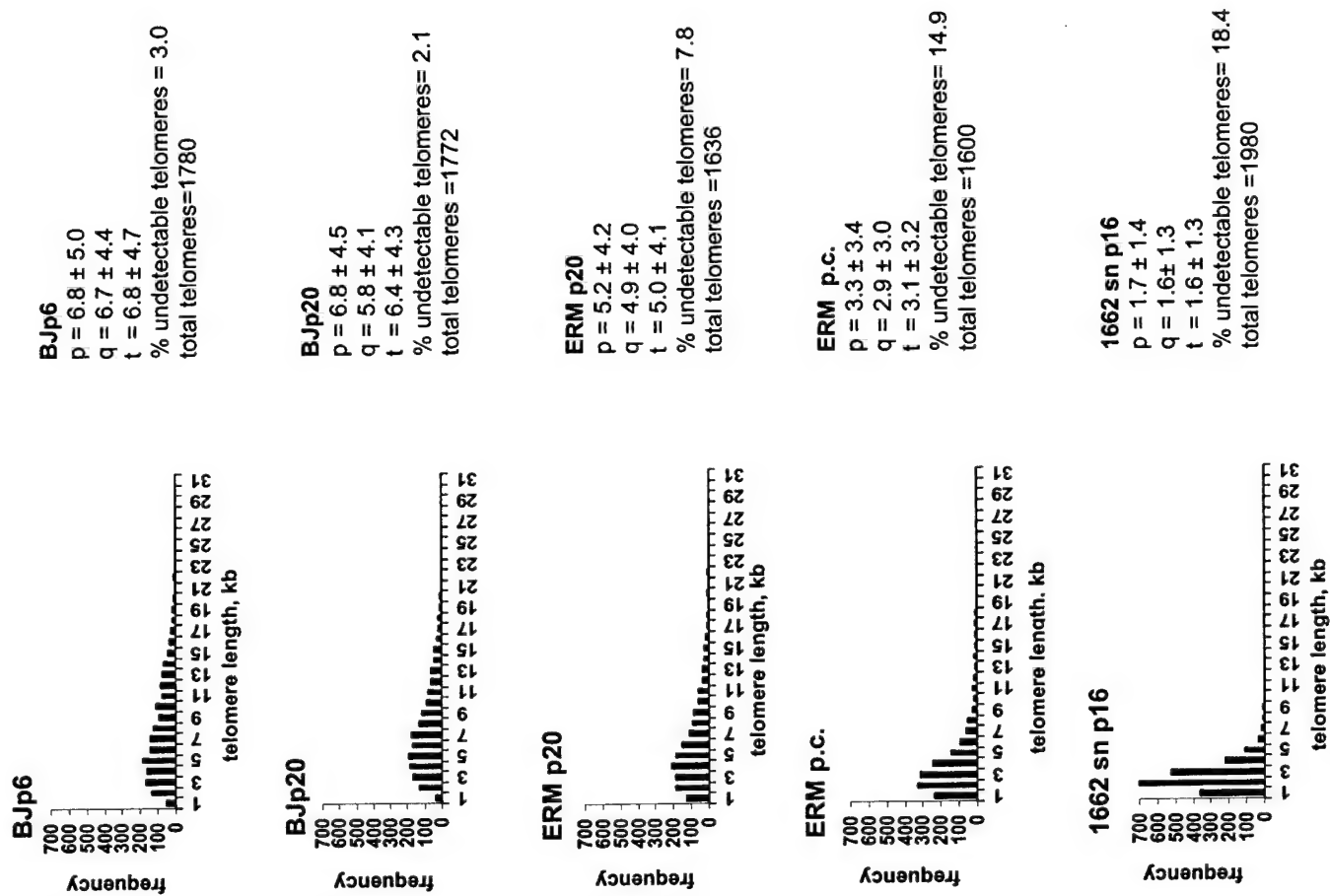| Cell Line	Number Tumors/Number Injected
293T	6/6
BJ	0/10
BJ/E1A + Ha-RasV12	0/6
BJ/E1A + MDM2	0/5
BJ/E1A + MDM2 + Ha-RasV12	34/48
BJ/E1A-143 + MDM2 + Ha-RasV12	6/6

Seger et al., Figure 4A and 4B

A.

BJp6 | BJp20 | BJp32 | ERMp20 | ERMp31 | ERM p.c.
2.3 1 +R | 2.3 1 +R | 2.3 1 +R | 2.3 1 +R | 2.3 1 +R | 2.3 1 +R

B.

293 cells + ERM tumor | ERM tumor | Human Tumor

Seger et al., Figure 4C

Seger et al., Figures 5A and 5B

B.

A.

Seger et al., Figure 5C

C.

BJp6
p = 6.8 ± 5.0
q = 6.7 ± 4.4
t = 6.8 ± 4.7
% undetectable telomeres = 3.0
total telomeres=1780

BJp20
p = 6.8 ± 4.5
q = 5.8 ± 4.1
t = 6.4 ± 4.3
% undetectable telomeres= 2.1
total telomeres =1772

ERM p20
p = 5.2 ± 4.2
q = 4.9 ± 4.0
t = 5.0 ± 4.1
% undetectable telomeres= 7.8
total telomeres =1636

ERM p.c.
p = 3.3 ± 3.4
q = 2.9 ± 3.0
t = 3.1 ± 3.2
% undetectable telomeres= 14.9
total telomeres =1600

1662 sn p16
p = 1.7 ± 1.4
q = 1.6± 1.3
t = 1.6 ± 1.3
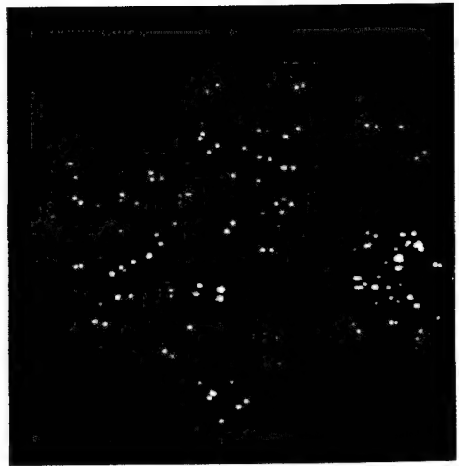% undetectable telomeres= 18.4
total telomeres =1980

Seger et al., Figure 5D

D.

BJp6

BJp20

ERM p20

ERM p.c.

1662 sn p16

Seger et al., Figure 6A

A.

BJ

BJ E1A/ras/mdm2

Seger et al., Figure 6B

B.

Seger et al., Figure 6C
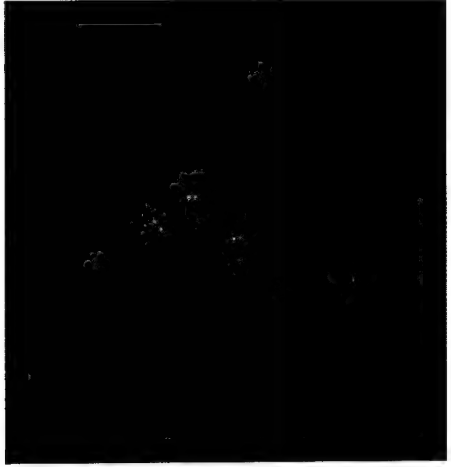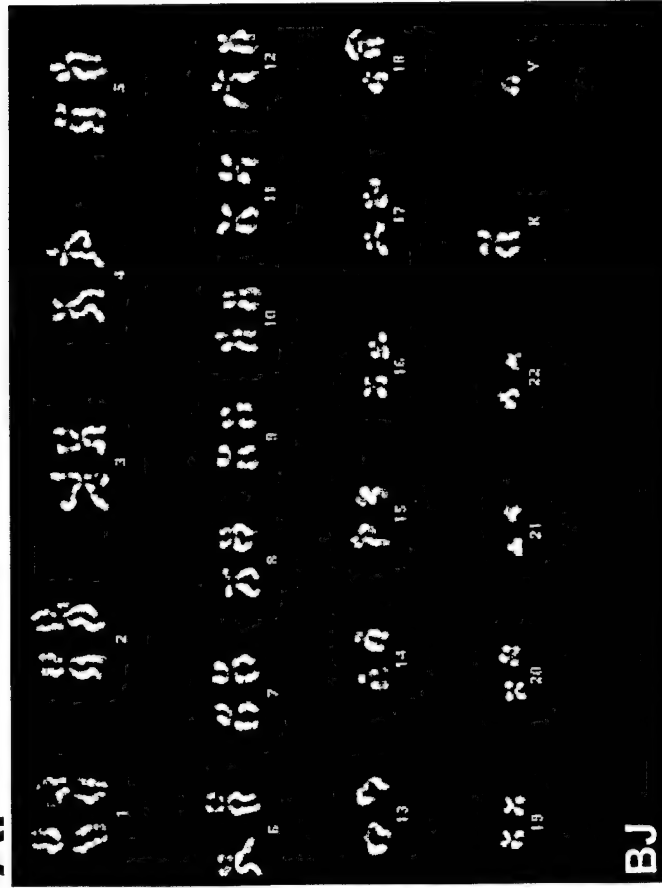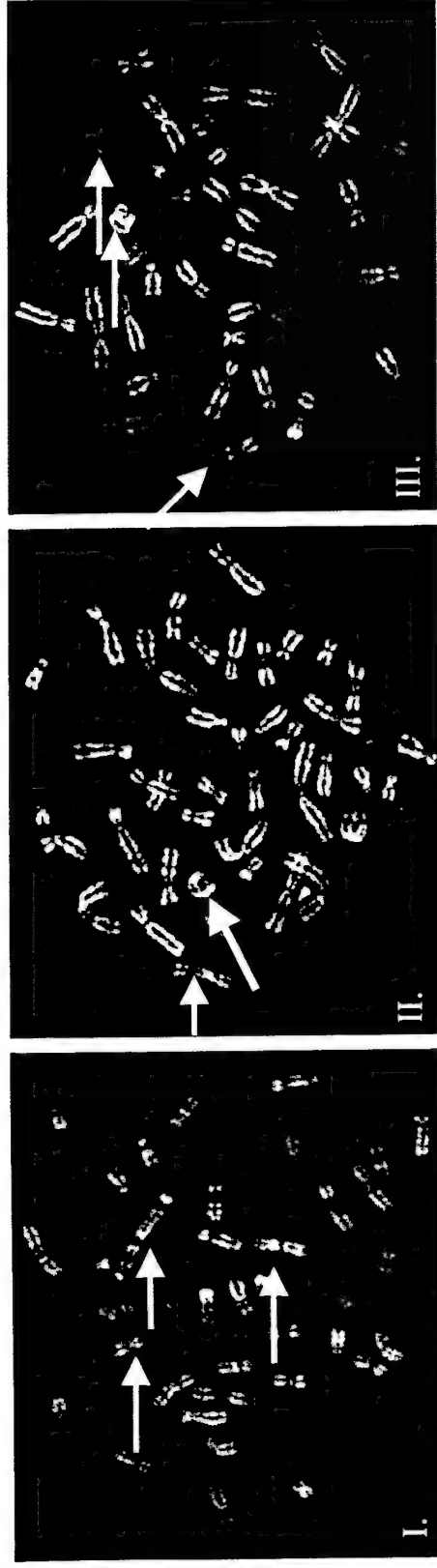
C.

	FRAGM.	DICENT.	TRICENT.	TRANSL.
BJ p6	0%	0%	0%	0%
BJ p20	0%	0%	0%	0%
ERM p20	0.16%	0.16%	0%	0%
ERM p.c.	2%	0.75%	0.5%	1.5%
1660 dx p16	0.67%	0.13%	0%	0%

ERM p.c.

TRANS.

DICENT.

TRICENT.

chromosome number vs karyotype number

Legend:
◆ BJ p6
■ BJ p20
ERM p20
× ERM p.c
✕ 1660 dx p16

Seger et al., Figure 7A and 7B
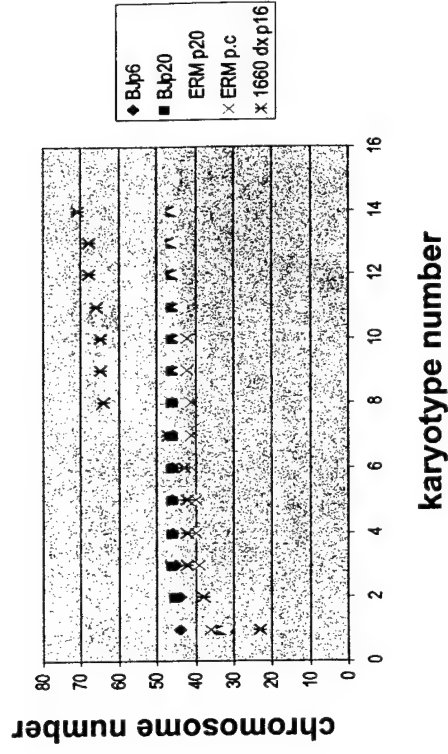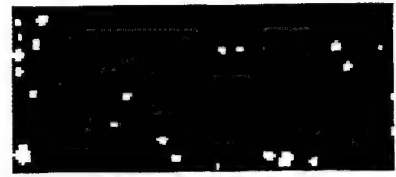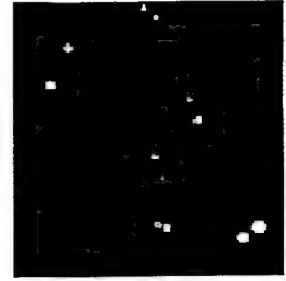
A.

BJ

HSF43

WI-38

B. Tumor Formation in Immunodeficient Mice: E1A + MDM2 + Ha-RasV12

Cell Line	Number Tumors/Number Injected
BJ/ERM	12/12
HSF43/ERM	2/8
WI-38/ERM	7/8
Detroit551/ERM	7/7

Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells

Patrick J. Paddison,[1] Amy A. Caudy,[1] Emily Bernstein,[2,3] Gregory J. Hannon,[1,2,4] and Douglas S. Conklin[2]

[1]Watson School of Biological Sciences, [2]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA; [3]Graduate Program in Genetics, State University of New York at Stony Brook, Stony Brook, New York 11794, USA

RNA interference (RNAi) was first recognized in *Caenorhabditis elegans* as a biological response to exogenous double-stranded RNA (dsRNA), which induces sequence-specific gene silencing. RNAi represents a conserved regulatory motif, which is present in a wide range of eukaryotic organisms. Recently, we and others have shown that endogenously encoded triggers of gene silencing act through elements of the RNAi machinery to regulate the expression of protein-coding genes. These small temporal RNAs (stRNAs) are transcribed as short hairpin precursors (~70 nt), processed into active, 21-nt RNAs by Dicer, and recognize target mRNAs via base-pairing interactions. Here, we show that short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in cultured *Drosophila* and mammalian cells. shRNAs can be synthesized exogenously or can be transcribed from RNA polymerase III promoters in vivo, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing.

An understanding of the biological role of any gene comes only after observing the phenotypic consequences of altering the function of that gene in a living cell or organism. In many cases, those organisms for which convenient methodologies for genetic manipulation exist blaze the trail toward an understanding of similar genes in less tractable organisms, such as mammals. The advent of RNA interference (RNAi) as an investigational tool has shown the potential to democratize at least one aspect of genetic manipulation, the creation of hypomorphic alleles, in organisms ranging from unicellular parasites (e.g., Shi et al. 2000) to mammals (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000).

Although *Caenorhabditis elegans* has, for some time, been well developed as a forward genetic system, the lack of methodologies for gene replacement by homologous recombination presented a barrier to assessing rapidly the consequences of loss of function in known genes. In an effort to overcome this limitation, Mello and Fire (Fire et al. 1998), building on earlier studies (Guo and Kemphues 1995), probed the utility of antisense RNA as a method for suppressing gene expression in worms. Through these efforts, they found that double-stranded RNA (dsRNA) was much more effective than antisense RNA as an inducer of gene silencing. Subsequent studies have shown that RNAi is a conserved biological response that is present in many, if not most, eukaryotic organisms (for review, see Bernstein et al. 2001b; Hammond et al. 2001b).

As a result of biochemical and genetic approaches in several experimental systems, the mechanisms underlying RNAi have begun to unfold (for review, see Bernstein et al. 2001b; Hammond et al. 2001b). These suggest the existence of a conserved machinery for dsRNA-induced gene silencing, which proceeds via a two-step mechanism. In the first step, the dsRNA silencing trigger is recognized by an RNase III family nuclease called Dicer, which cleaves the dsRNA into ~21–23-nt siRNAs (small interfering RNAs). These siRNAs are incorporated into a multicomponent nuclease complex, RISC, which identifies substrates through their homology to siRNAs and targets these cognate mRNAs for destruction.

Although it was clear from the outset that RNAi would prove a powerful tool for manipulating gene expression in invertebrates, there were several potential impediments to the use of this approach in mammalian cells. Most mammalian cells harbor a potent antiviral response that is triggered by the presence of dsRNA viral

replication intermediates. A key component of this response is a dsRNA-activated protein kinase, PKR, which phosphorylates EIF-2α, inducing, in turn, a generalized inhibition of translation (for review, see Williams 1997; Gil and Esteban 2000). In addition, dsRNA activates the 2'5' oligoadenylate polymerase/RNase L system and represses IκB. The ultimate outcome of this set of responses is cell death via apoptosis.

Therefore, it came as a welcome surprise that dsRNA could induce sequence-specific silencing in mammalian embryos, which apparently lack generalized responses to dsRNA (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). Indeed, microinjection of dsRNA into mouse zygotes could specifically silence both exogenous reporters and endogenous genes to create anticipated phenotypes. Subsequently, these observations were extended to embryonic cell lines, such as embryonic stem cells and embryonal carcinoma cells, which do not show generic translational repression in response to dsRNA (Billy et al. 2001; Yang et al. 2001; Paddison et al. 2002). However, restriction of conventional RNAi to these few embryonic and cell culture systems would place a significant limitation on the utility of this approach in mammals.

Tuschl and colleagues first showed that short RNA duplexes, designed to mimic the products of the Dicer enzyme, could trigger RNA interference in vitro in Drosophila embryo extracts (Tuschl et al. 1999; Elbashir et al. 2001b,c). This observation was extended to mammalian somatic cells by Tuschl and coworkers (Elbashir et al. 2001a) and by Fire and colleagues (Caplen et al. 2001), who showed that chemically synthesized siRNAs could induce gene silencing in a wide range of human and mouse cell lines. The use of synthetic siRNAs to transiently suppress the expression of target genes is quickly becoming a method of choice for probing gene function in mammalian cells.

Dicer, the enzyme that normally produces siRNAs in vivo, has been linked to RNA interference both through biochemistry and through genetics (Bernstein et al. 2001a; Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001). Indeed, C. elegans animals that lack Dicer are RNAi-deficient, at least in some tissues. However, these animals also have additional phenotypic abnormalities. Specifically, they are sterile and show a number of developmental abnormalities that typify alterations in developmental timing. Indeed, the phenotypes of the Dicer mutant animals were similar to those previously observed for animals carrying mutations in the let-7 gene (Reinhart et al. 2000).

The let-7 gene encodes a small, highly conserved RNA species that regulates the expression of endogenous protein-coding genes during worm development. The active RNA species is transcribed initially as an ~70-nt precursor, which is posttranscriptionally processed into a mature ~21-nt form (Reinhart et al. 2000). Both in vitro and in vivo data from C. elegans (Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001) and human cells (Hutvagner et al. 2001) have pointed to Dicer as the enzyme responsible for let-7 maturation and for the matu-

ration of a similar small RNA, lin-4 (Grishok et al. 2001). Thus, at least some components of the RNAi machinery respond to endogenously encoded triggers to regulate the expression of target genes.

Recent studies have placed let-7 and lin-4 as the founding members of a potentially very large group of small RNAs known generically as micro-RNAs (miRNAs). Nearly 100 potential miRNAs have now been identified in Drosophila, C. elegans, and mammals (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). Although the functions of these diverse RNAs remain mysterious, it seems likely that they, like let-7 and lin-4, are transcribed as hairpin RNA precursors, which are processed to their mature forms by Dicer (Lee and Ambros 2001; E. Bernstein, unpubl.).

Since the realization that small, endogenously encoded hairpin RNAs could regulate gene expression via elements of the RNAi machinery, we have sought to exploit this biological mechanism for the regulation of desired target genes. Here we show that short hairpin RNAs (shRNAs) can induce sequence-specific gene silencing in mammalian cells. As is normally done with siRNAs, silencing can be provoked by transfecting exogenously synthesized hairpins into cells. However, silencing can also be triggered by endogenous expression of shRNAs. This observation opens the door to the production of continuous cells lines in which RNAi is used to stably suppress gene expression in mammalian cells. Furthermore, similar approaches should prove efficacious in the creation of transgenic animals and potentially in therapeutic strategies in which long-term suppression of gene function is essential to produce a desired effect.

Results

Short hairpin RNAs trigger gene silencing in Drosophila cells

Several groups (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) have shown that endogenous triggers of gene silencing, specifically small temporal RNAs (stRNAs) let-7 and lin-4, function at least in part through RNAi pathways. Specifically, these small RNAs are encoded by hairpin precursors that are processed by Dicer into mature, ~21-nt forms. Moreover, genetic studies in C. elegans have shown a requirement for Argonaute-family proteins in stRNA function. Specifically, alg-1 and alg-2, members of the EIF2c subfamily, are implicated both in stRNA processing and in their downstream effector functions (Grishok et al. 2001). We have recently shown that a component of RISC, the effector nuclease of RNAi, is a member of the Argonaute family, prompting a model in which stRNAs may function through RISC-like complexes, which regulate mRNA translation rather than mRNA stability (Hammond et al. 2001a).

We wished to test the possibility that we might retarget these small, endogenously encoded hairpin RNAs to regulate genes of choice with the ultimate goal of sub-

verting this regulatory system for manipulating gene expression stably in mammalian cell lines and in transgenic animals. Whether triggered by long dsRNAs or by siRNAs, RNAi is generally more potent in the suppression of gene expression in *Drosophila* S2 cells than in mammalian cells. We therefore chose this model system in which to test the efficacy of short hairpin RNAs (shRNAs) as inducers of gene silencing.

Neither stRNAs nor the broader group of miRNAs that has recently been discovered form perfect hairpin structures. Indeed, each of these RNAs is predicted to contain several bulged nucleotides within their rather short (~30-nt) stem structures. Because the position and character of these bulged nucleotides have been conserved throughout evolution and among at least a subset of miRNAs, we sought to design retargeted miRNA mimics to conserve these predicted structural features. Only the *let-7* and *lin-4* miRNAs have known mRNA targets (Wightman et al. 1993; Slack et al. 2000). In both cases, pairing to binding sites within the regulated transcripts is imperfect, and in the case of *lin-4*, the presence of a bulged nucleotide is critical to suppression (Ha et al. 1996). We therefore also designed shRNAs that paired

imperfectly with their target substrates. A subset of these shRNAs is depicted in Figure 1A.

To permit rapid testing of large numbers of shRNA variants and quantitative comparison of the efficacy of suppression, we chose to use a dual-luciferase reporter system, as previously described for assays of RNAi in both *Drosophila* extracts (Tuschl et al. 1999) and mammalian cells (Caplen et al. 2001; Elbashir et al. 2001a). Cotransfection of firefly and *Renilla* luciferase reporter plasmids with either long dsRNAs or with siRNAs homologous to the firefly luciferase gene yielded an ~95% suppression of firefly luciferase without effect on *Renilla* luciferase (Fig. 1B; data not shown). Firefly luciferase could also be specifically silenced by cotransfection with homologous shRNAs. Surprisingly, those shRNAs modeled most closely on the *let-7* paradigm were the least effective inducers of silencing (data not shown). The inclusion of bulged nucleotides within the shRNA stem caused only a modest reduction in potency; however, the presence of mismatches with respect to the target mRNA essentially abolished silencing potential. The most potent inhibitors were those composed of simple hairpin structures with complete homology to the sub-

Figure 1. Short hairpins suppress gene expression in *Drosophila* S2 cells. (A) Sequences and predicted secondary structure of representative chemically synthesized RNAs. Sequences correspond to positions 112–134 (siRNA) and 463–491 (shRNAs) of Firefly luciferase carried on pGL3-Control. An siRNA targeted to position 463–485 of the luciferase sequence was virtually identical in suppressing expression, but is not shown. (B) Exogenously supplied short hairpins suppress expression of the targeted Firefly luciferase gene in vivo. Six-well plates of S2 cells were transfected with 250 ng/well of plasmids that direct the expression of firefly and *Renilla* luciferase and 500 ng/well of the indicated RNA. Luciferase activities were assayed 48 h after transfection. Ratios of firefly to *Renilla* luciferase activity were normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation. (C) Short hairpins are processed by the *Drosophila* Dicer enzyme. T7 transcribed hairpins shFfL22, shFfL29, and shFfS29 were incubated with (+) and without (–) 0–2-h *Drosophila* embryo extracts. Those incubated with extract produced ~22-nt siRNAs, consistent with the ability of these hairpins to induce RNA interference. A long dsRNA input (cyclin E 500-mer) was used as a control. Cleavage reactions were performed as described in Bernstein et al. (2001a).
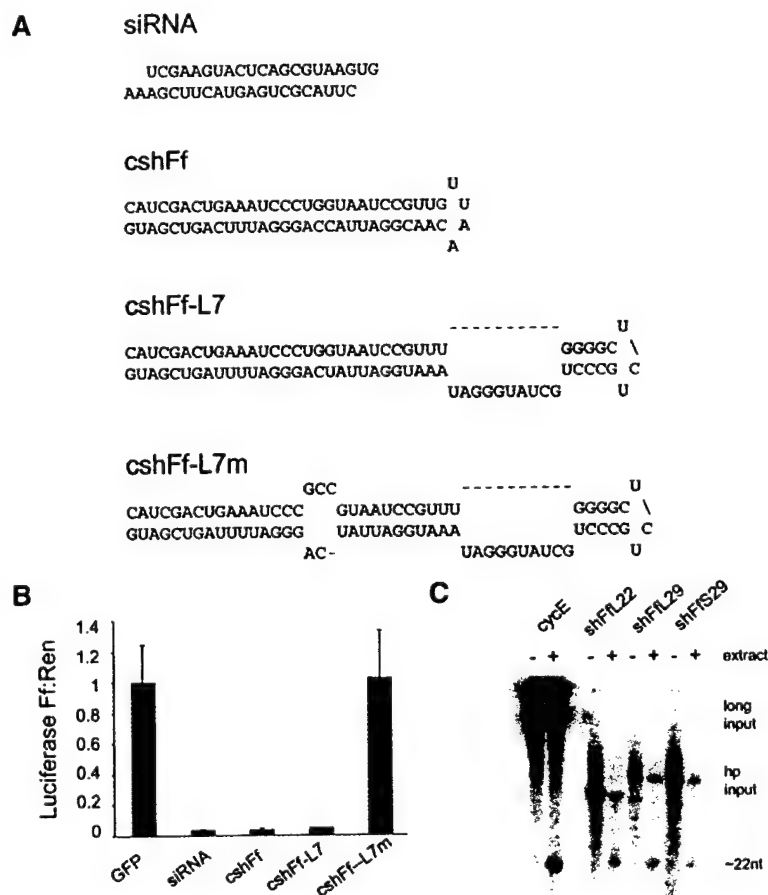
strate. Introduction of G-U basepairs either within the stem or within the substrate recognition sequence had little or no effect (Fig. 1A,B; data not shown). Similarly, varying either the loop size from ~4 to 23 bases or the loop sequence (e.g., to mimic *let-7*) also proved neutral (data not shown).

These results show that short hairpin RNAs can induce gene silencing in *Drosophila* S2 cells with potency similar to that of siRNAs (Fig. 1B). However, in our initial observation of RNA interference in *Drosophila* S2 cells, we noted a profound dependence of the efficiency of silencing on the length of the dsRNA trigger (Hammond et al. 2000). Indeed, dsRNAs of fewer than ~200 nt triggered silencing very inefficiently. Silencing is initiated by an RNase III family nuclease, Dicer, that processes long dsRNAs into ~22-nt siRNAs. In accord with their varying potency as initiators of silencing, long dsRNAs are processed much more readily into short RNAs by the Dicer enzyme (Bernstein et al. 2001a). We therefore tested whether shRNAs were substrates for the Dicer enzyme.

We had noted previously that *let-7* (Ketting et al. 2001) and other miRNAs (E. Bernstein, unpubl.) are processed by Dicer with an unexpectedly high efficiency as compared with short, nonhairpin dsRNAs. Similarly, Dicer efficiently processed shRNAs that targeted firefly luciferase, irrespective of whether they were designed to mimic a natural Dicer substrate (*let-7*) or whether they were simple hairpin structures (Fig. 1C). These data suggest that recombinant shRNAs can be processed by Dicer into siRNAs and are consistent with the idea that these short hairpins trigger gene silencing via an RNAi pathway.

Short hairpin activated gene silencing in mammalian cells

RNAi is developing into an increasingly powerful methodology for manipulating gene expression in diverse experimental systems. However, mammalian cells contain several endogenous systems that were predicted to hamper the application of RNAi. Chief among these is a dsRNA-activated protein kinase, PKR, which effects a general suppression of translation via phosphorylation of EIF-2α (Williams 1997; Gil and Esteban 2000). Activation of these, and other dsRNA-responsive pathways, generally requires duplexes exceeding 30 bp in length, possibly to permit dimerization of the enzyme on its allosteric activator (e.g., Clarke and Mathews 1995).

Small RNAs that mimic Dicer products, siRNAs, presumably escape this limit and trigger specific silencing, in part because of their size. However, short duplex RNAs that lack signature features of siRNAs can efficiently induce silencing in *Drosophila* S2 cells but not in mammalian cells (A.A. Caudy, unpubl.). Endogenously encoded miRNAs may also escape PKR surveillance because of their size but perhaps also because of the discontinuity of their duplex structure. Given that shRNAs of <30 bp were effective inducers of RNAi in *Drosophila*

S2 cells, we tested whether these RNAs could also induce sequence-specific silencing in mammalian cells.

Human embryonic kidney (HEK293T) cells were co-transfected with chemically synthesized shRNAs and with a mixture of firefly and *Renilla* luciferase reporter plasmids. As had been observed in S2 cells, shRNAs were effective inducers of gene silencing. Once again, hairpins designed to mimic *let-7* were consistently less effective than were simple hairpin RNAs, and the introduction of mismatches between the antisense strand of the shRNA and the mRNA target abolished silencing (Fig. 2A; data not shown). Overall, shRNAs were somewhat less potent silencing triggers than were siRNAs. Whereas siRNAs homologous to firefly luciferase routinely yielded ~90%–95% suppression of gene expression, suppression levels achieved with shRNAs ranged from 80%–90% on average. As we also observe with siRNAs, the most important determinant of the potency of the silencing trigger is its sequence. We find that roughly 50% of both siRNAs and shRNAs are competent for suppressing gene expression. However, neither analysis of the predicted structures of the target mRNA nor analysis of alternative structures in siRNA duplexes or shRNA hairpins has proved of predictive value for choosing effective inhibitors of gene expression.

We have adopted as a standard, shRNA duplexes containing 29 bp. However, the size of the helix can be reduced to ~25 nt without significant loss of potency. Duplexes as short as 22 bp can still provoke detectable silencing, but do so less efficiently than do longer duplexes. In no case do we observe a reduction in the internal control reporter (*Renilla* luciferase) that would be consistent with an induction of nonspecific dsRNA responses.

The ability of shRNAs to induce gene silencing was not confined to 293T cells. Similar results were also obtained in a variety of other mammalian cell lines, including human cancer cells (HeLa), transformed monkey epithelial cells (COS-1), murine fibroblasts (NIH 3T3), and diploid human fibroblasts (IMR90; Fig. 2; data not shown).

Synthesis of effective inhibitors of gene expression using T7 RNA polymerase

The use of siRNAs to provoke gene silencing is developing into a standard methodology for investigating gene function in mammalian cells. To date, siRNAs have been produced exclusively by chemical synthesis (e.g., Caplen et al. 2001; Elbashir et al. 2001a). However, the costs associated with this approach are significant, limiting its potential utility as a tool for investigating in parallel the functions of large numbers of genes. Short hairpin RNAs are presumably processed into active siRNAs in vivo by Dicer (see Fig. 1C). Thus, these may be more tolerant of terminal structures, both with respect to nucleotide overhangs and with respect to phosphate termini. We therefore tested whether shRNAs could be prepared by in vitro transcription with T7 RNA polymerase.
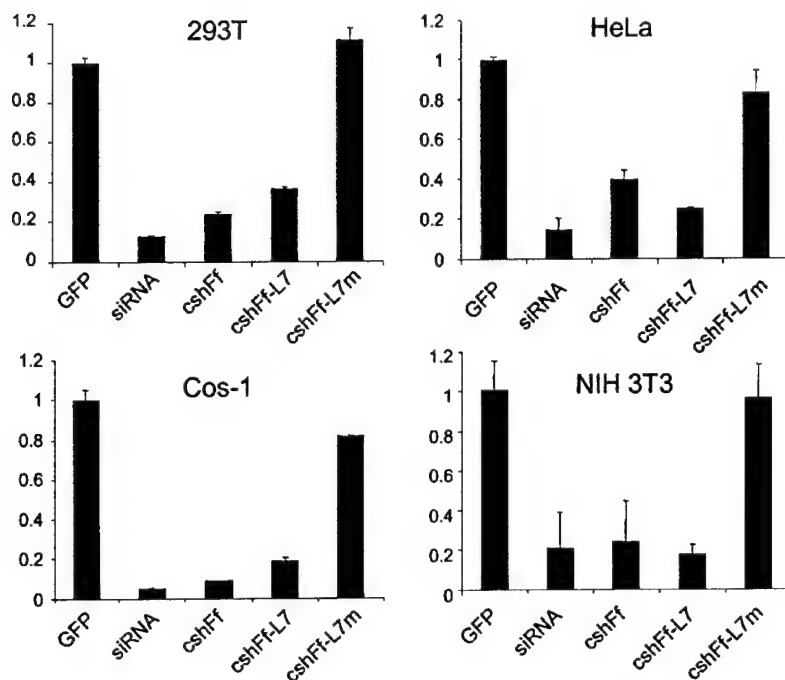
Figure 2. Short hairpins function in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with plasmids and RNAs as in Figure 1 and subjected to dual luciferase assays 48 h posttransfection. The ratios of firefly to *Renilla* luciferase activity are normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation.

Transcription templates that were predicted to generate siRNAs and shRNAs similar to those prepared by chemical RNA synthesis were prepared by DNA synthesis (Fig. 3A,C). These were tested for efficacy both in S2 cells (data not shown) and in human 293 cells (Fig. 3B,D). Overall, the performance of the T7-synthesized hairpin or siRNAs closely matched the performance of either produced by chemical synthesis, both with respect to the magnitude of inhibition and with respect to the relative efficiency of differing sequences. Because T7 polymerase prefers to initiate at twin guanosine residues, however, it was critical to consider initiation context when designing in vitro transcribed siRNAs (Fig. 3B). In contrast, shRNAs, which are processed by Dicer (see Fig. 1C), tolerate the addition of these bases at the 5' end of the transcript.

Studies in *Drosophila* embryo extracts indicate that siRNAs possess 5' phosphorylated termini, consistent with their production by an RNase III family nuclease (Bernstein et al. 2001a; Elbashir et al. 2001b). In vitro, this terminus is critical to the induction of RNAi by synthetic RNA oligonucleotides (Elbashir et al. 2001c; Nykanen et al. 2001). Chemically synthesized siRNAs are nonphosphorylated, and enzymatic addition of a 5' phosphate group in vitro prior to transfection does not increase the potency of the silencing effect (A.A. Caudy, unpubl.). This suggests either that the requirement for phosphorylated termini is less stringent in mammalian

cells or that a kinase efficiently phosphorylates siRNAs in vivo. RNAs synthesized with T7 RNA polymerase, however, possess 5' triphosphate termini. We therefore explored the possibility of synthesizing siRNAs with T7 polymerase followed by treatment in vitro with pyrophosphatase to modify the termini to resemble those of siRNAs. Surprisingly, monophosphorylated siRNAs (data not shown) were as potent in inducing gene silencing as transcription products bearing triphosphate termini (Fig. 3B). This may suggest either that the requirement for monophosphorylated termini is less stringent in mammalian cells or that siRNAs are modified in vivo to achieve an appropriate terminal structure.

Considered together, our data suggest that both shRNAs and siRNA duplexes can be prepared by synthesis with T7 RNA polymerase in vitro. This significantly reduces the cost of RNAi in mammalian cells and paves the way for application of RNAi on a whole-genome scale.

Transcription of shRNAs in vivo by RNA polymerase III

Although siRNAs are an undeniably effective tool for probing gene function in mammalian cells, their suppressive effects are by definition of limited duration. Delivery of siRNAs can be accomplished by any of a num-

A siRNA

UCGAAGUACUCAGCGUAAGUG
AAAGCUUCAUGAGUCGCAUUC

T7siRNA

GGUCGAAGUACUCAGCGUAAGAA
AAAGCUUCAUGAGUCGCAUUCGG

T7siFf-2

GGUUGUGGAUCUGGAUACCGG
UUCCAACACCUAGACCUAUGG

T7siFf-3

GGUGCCAACCCUAUUCUCCUU
GACCACGGUUGGGAUAAGAGG

T7siFf-8

GGCUAUGAAGAGAGUACGCCCU
UUCCGAUACUUCUCUCAUGCGG

B

C T7shFf29

```
GGU|                                    U
  CGAAGUACUCAGCGUAAGUGAUGUCCAC U
  GUUUUGUGGGUUGUGUUUGUUGUGGGUG A
G^                                      A
```

T7shFf27

```
GGU|                                U
  CGAAGUACUCAGCGUAAGUGAUGUCC U
  GUUUUGUGGGUUGUGUUUGUUGUGGG A
G^                                  A
```
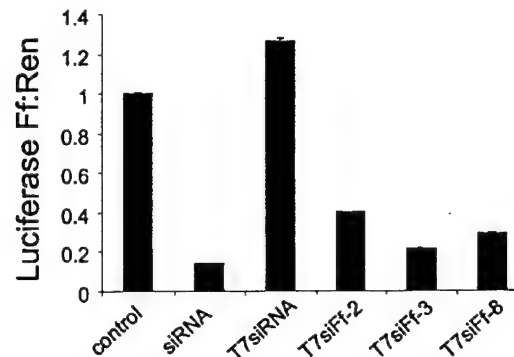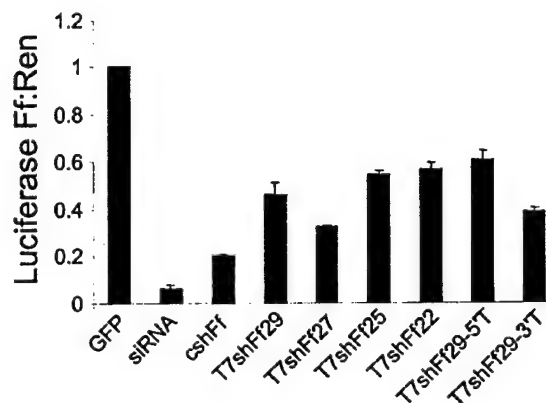
T7shFf25

```
GGU|                            U
  CGAAGUACUCAGCGUAAGUGAUGU U
  GUUUUGUGGGUUGUGUUUGUUGUG A
G^                              A
```

T7shFf22

```
GGU|                        U
  CGAAGUACUCAGCGUAAGUGA U
  GUUUUGUGGGUUGUGUUUGUU A
G^                          A
```

T7shFf29-5'T

```
GGCUCGAGU|                              U
        CGAAGUACUCAGCGUAAGUGAUGUCCAC U
        GUUUUGUGGGUUGUGUUUGUUGUGGGUG A
G--------^                              A
```

T7shFf29-3'T

```
-----G|                                U
     GUCGAAGUACUCAGCGUAAGUGAUGUCCAC U
     CGGUUUUGUGGGUUGUGUUUGUUGUGGGUG A
GAGCU^                                  A
```

D

Figure 3. siRNAs and short hairpins transcribed in vitro suppress gene expression in mammalian cells. (A) Sequences and predicted secondary structure of representative in vitro transcribed siRNAs. Sequences correspond to positions 112–134 (siRNA) and 463–491 (shRNAs) of firefly luciferase carried on pGL3-Control. (B) In vitro transcribed siRNAs suppress expression of the targeted firefly luciferase gene in vivo. HEK 293T cells were transfected with plasmids as in Figure 2. The presence of non-base-paired guanosine residues at the 5' end of siRNAs significantly alters the predicted end structure and abolishes siRNA activity. (C) Sequences and predicted secondary structure of representative in vitro transcribed shR-NAs. Sequences correspond to positions 112–141 of firefly luciferase carried on pGL3-Control. (D) Short hairpins transcribed in vitro suppress expression of the targeted firefly luciferase gene in vivo. HEK 293T cells were transfected with plasmids as in Figure 2.

ber of transient transfection methodologies, and both the timing of peak suppression and the recovery of protein levels as silencing decays can vary with both the cell type and the target gene (Y. Seger and E. Bernstein, unpubl.). Therefore, one limitation on siRNAs is the devel-opment of continuous cell lines in which the expression of a desired target is stably silenced.

Hairpin RNAs, consisting of long duplex structures, have been proved as effective triggers of stable gene silencing in plants, in *C. elegans*, and in *Drosophila* (Ken-

nerdell and Carthew 2000; Smith et al. 2000; Tavernarakis et al. 2000). We have recently shown stable suppression of gene expression in cultured mammalian cells by continuous expression of a long hairpin RNA (Paddison et al. 2002). However, the scope of this approach was limited by the necessity of expressing such hairpins only in cells that lack a detectable PKR response. In principle, shRNAs could bypass such limitations and provide a tool for evoking stable suppression by RNA in mammalian somatic cells.

To test this possibility, we initially cloned sequences encoding a firefly luciferase shRNA into a CMV-based expression plasmid. This was predicted to generate a capped, polyadenylated RNA polymerase II transcript in which the hairpin was extended on both the 5' and 3' ends by vector sequences and poly(A). This construct was completely inert in silencing assays in 293T cells (data not shown).

During our studies on chemically and T7-synthesized shRNAs, we noted that the presence of significant single-stranded extensions (either 5' or 3' of the duplex) reduced the efficacy of shRNAs (data not shown). We therefore explored the use of alternative promoter strategies in an effort to produce more defined hairpin RNAs. In particular, RNA polymerase III promoters have well-defined initiation and termination sites and naturally produce a variety of small, stable RNA species. Although many Pol III promoters contain essential elements within the transcribed region, limiting their utility for our purposes; class III promoters use exclusively non-transcribed promoter sequences. Of these, the U6 snRNA promoter and the H1 RNA promoter have been well studied (Lobo et al. 1990; Hannon et al. 1991; Chong et al. 2001).

By placing a convenient cloning site immediately behind the U6 snRNA promoter, we have constructed pShh-1, an expression vector in which short hairpins are harnessed for gene silencing. Into this vector either of two shRNA sequences derived from firefly luciferase were cloned from synthetic oligonucleotides. These were cotransfected with firefly and *Renilla* luciferase expression plasmids into 293T cells. One of the two encoded shRNAs provoked effective silencing of firefly luciferase without altering the expression of the internal control (Fig. 4C). The second encoded shRNA also produced detectable, albeit weak, repression. In both cases, silencing was dependent on insertion of the shRNA in the correct orientation with respect to the promoter (Fig. 4C; data not shown). Although the shRNA itself is bilaterally symmetric, insertion in the incorrect orientation would affect Pol III termination and is predicted to produce a hairpin with both 5' and 3' single-stranded extensions. Similar results were also obtained in a number of other mammalian cell lines including HeLa, COS-1, NIH 3T3, and IMR90 (Fig. 4; data not shown). pShh1-Ff1 was, however, incapable of effecting suppression of the luciferase reporter in *Drosophila* cells, in which the human U6 promoter is inactive (data not shown).

As a definitive test of whether the plasmid-encoded shRNAs brought about gene silencing via the mamma-

lian RNAi pathway, we assessed the dependence of suppression on an essential component of the RNAi pathway. We transfected pShh1-Ff1 along with an siRNA homologous to human *Dicer*. Figure 5 shows that treatment of cells with *Dicer* siRNAs is able to completely depress the silencing induced by pShh1-Ff1. Addition of an unrelated siRNA had no effect on the magnitude of suppression by pShh1-Ff1 (data not shown). Importantly, *Dicer* siRNAs had no effect on siRNA-induced silencing of firefly luciferase (data not shown). These results are consistent with shRNAs operating via an RNAi pathway similar to those provoked by stRNAs and long dsRNAs. Furthermore, it suggests that siRNA-mediated silencing is less sensitive to depletion of the Dicer enzyme.

The ultimate utility of encoded short hairpins will be in the creation of stable mutants that permit the study of the resulting phenotypes. We therefore tested whether we could create a cellular phenotype through stable suppression. Expression of activated alleles of the *ras* oncogene in primary mouse embryo fibroblasts (MEFs) induces a stable growth arrest that resembles, as a terminal phenotype, replicative senescence (Serrano et al. 1997). Cells cease dividing and assume a typical large, flattened morphology. Senescence can be countered by mutations that inactivate the p53 tumor suppressor pathway (Serrano et al. 1997). As a test of the ability of vector-encoded shRNAs to stably suppress an endogenous cellular gene, we generated a hairpin that was targeted to the mouse *p53* gene. As shown in Figure 6, MEFs transfected with pBabe-RasV12 fail to proliferate and show a senescent morphology when cotransfected with an empty control vector. As noted previously (Serrano et al. 1997), the terminally arrested state is achieved in 100% of drug-selected cells in culture by 8 d posttransfection. However, upon cotransfection of an activated *ras* expression construct with the pShh-p53, cells emerged from drug selection that not only fail to adopt a senescent morphology but also maintain the ability to proliferate for a minimum of several weeks in culture (Fig. 6). These data strongly suggest that shRNA expression constructs can be used for the creation of continuous mammalian cell lines in which selected target genes are stably suppressed.

Discussion

The demonstration that short dsRNA duplexes can induce sequence-specific silencing in mammalian cells has begun to foment a revolution in the manner in which gene function is examined in cultured mammalian cells. These siRNAs (Elbashir et al. 2001a) mimic the products generated by Dicer (Bernstein et al. 2001a) in the initiation step of RNAi and presumably enter the silencing pathway without triggering nonspecific translational suppression via PKR. siRNAs can be used to examine the consequences of reducing the function of virtually any protein-coding gene and have proved effective in provoking relevant phenotypes in numerous somatic cell types from both humans and mice. However, a significant dis-
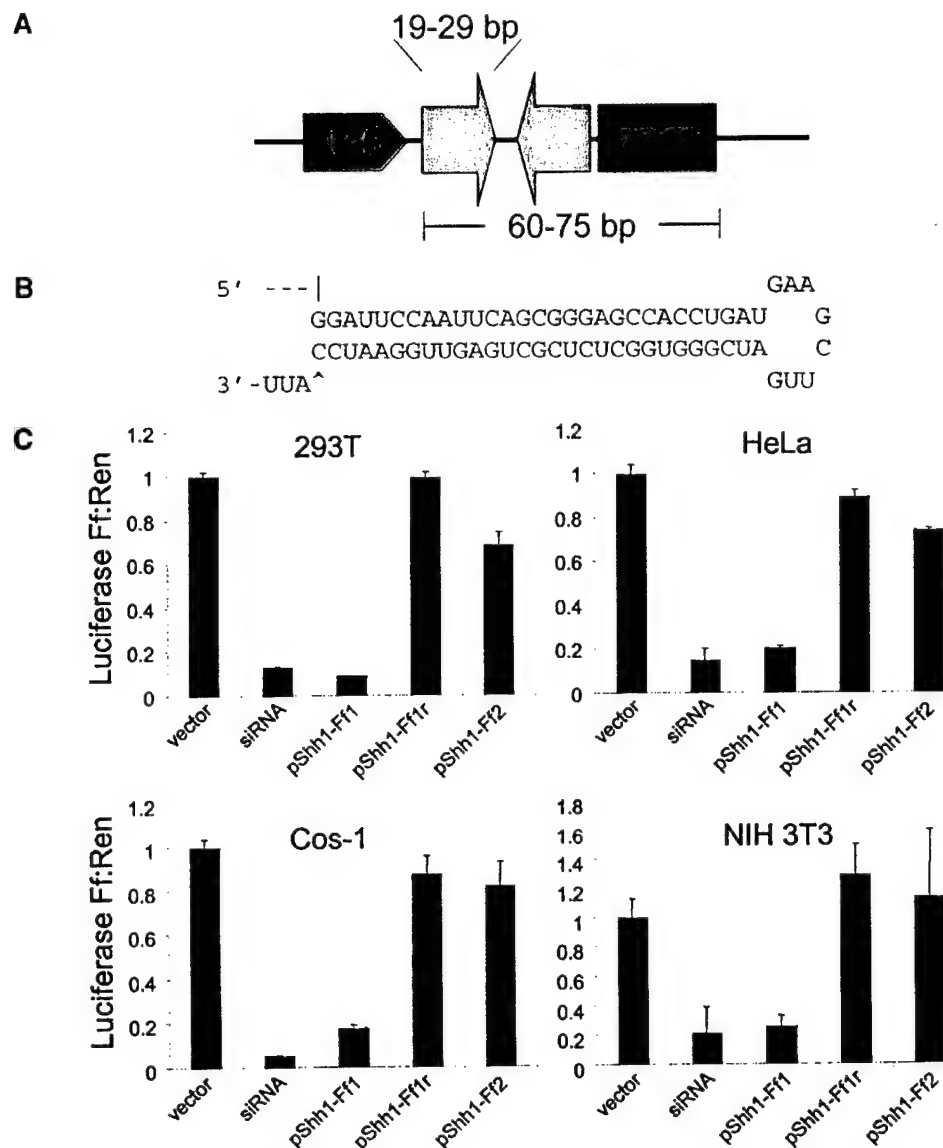
Figure 4. Transcription of functional shRNAs in vivo. (A) Schematic of the pShh1 vector. Sequences encoding shRNAs with between 19 and 29 bases of homology to the targeted gene are synthesized as 60–75-bp double-stranded DNA oligonucleotides and ligated into an EcoRV site immediately downstream of the U6 promoter. (B) Sequence and predicted secondary structure of the Ff1 hairpin. (C) An shRNA expressed from the pShh1 vector suppresses luciferase expression in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with reporter plasmids as in Figure 1, and pShh1 vector, firefly siRNA, or pShh1 firefly shRNA constructs as indicated. The ratios of firefly to Renilla luciferase activity were determined 48 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

advantage of siRNAs is that their effects are transient, with phenotypes generated by transfection with such RNAs persisting for ~1 wk. In *C. elegans*, RNAi has proved to be such a powerful tool, in part, because silencing is both systemic and heritable, permitting the consequences of altering gene expression to be examined

throughout the development and life of an animal. We have therefore sought to expand the utility of RNAi in mammalian systems by devising methods to induce stable and heritable gene silencing. Previously, we have shown that expression of long (~500-nt) dsRNAs could produce stable silencing in embryonic mammalian cells
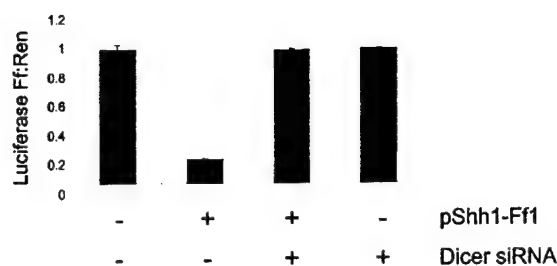
Figure 5. Dicer is required for shRNA-mediated gene silencing. HEK 293T cells were transfected with luciferase reporter plasmids as well as pShh1-Ff1 and an siRNA targeting human Dicer either alone or in combination, as indicated. The Dicer siRNA sequence (TCA ACC AGC CAC TGC TGG A) corresponds to coordinates 3137–3155 of the human *Dicer* sequence. The ratios of firefly to *Renilla* luciferase activity were determined 26 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

(Paddison et al. 2002); however, the utility of this approach was limited by its restriction to cells that lack endogenous, nonspecific responses to dsRNA, such as PKR.

Recently, a number of laboratories (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) have shown that there exist endogenously encoded triggers of RNAi-related pathways, which are transcribed as short hairpin RNAs (stRNAs, or generically miRNAs). Here, we have shown that short hairpin

RNAs, modeled conceptually on miRNAs, are potent experimental tools for inducing gene silencing in mammalian somatic cells. These shRNAs can be provided exogenously or can be synthesized in vivo from RNA polymerase III promoters. Not only does this enable the creation of continuous cell lines in which suppression of a target gene is stably maintained by RNAi, but similar strategies may also be useful for the construction of transgenic animals. Thus, short-hairpin-activated gene silencing (SHAGging) provides a complement to the use of siRNAs in the study of gene function in mammalian cells. Finally, the ability to encode a constitutive silencing signal may permit the marriage of shRNA-induced silencing with in vivo and ex vivo gene delivery methods for therapeutic approaches based on stable RNAi in humans.
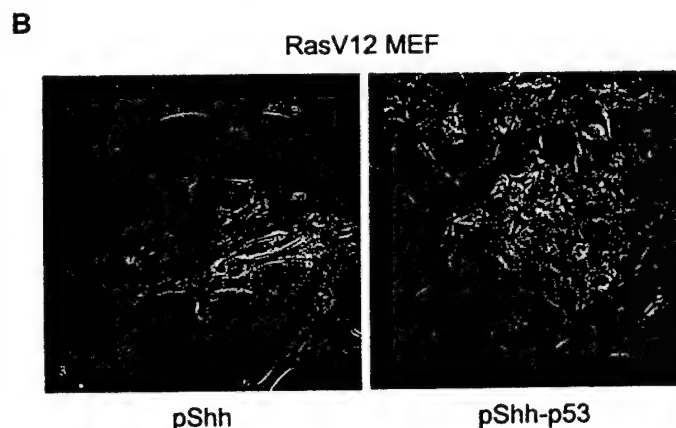
Materials and methods

Cell culture

HEK 293T, HeLa, COS-1, MEF, and IMR90 cells were cultured in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (GIBCO BRL). NIH 3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated calf serum and 1% antibiotic/antimycotic solution.

RNA preparation

Both shRNAs and siRNAs were produced in vitro using chemically synthesized DNA oligonucleotide templates (Sigma) and the T7 Megashortscript kit (Ambion). Transcription templates
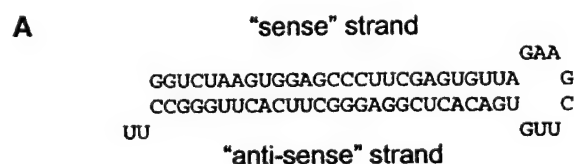
Figure 6. Stable shRNA-mediated gene silencing of an endogenous gene. (*A*) Sequence and predicted secondary structure of the *p53* hairpin. The 5′ shRNA stem contains a 27-nt sequence derived from mouse *p53* (nucleotides 166–192), whereas the 3′ stem harbors the complimentary antisense sequence. (*B*) Senescence bypass in primary mouse embryo fibroblasts (MEFs) expressing an shRNA targeted at *p53*. Wild-type MEFs, passage 5, were transfected with pBabe-RasV12 with control plasmid or with p53hp (5 μg each with FuGENE; Roche). Two days after transfection, cells were trypsinized, counted, and plated at a density of 1×10^5/10-cm plate in media containing 2.0 μg/mL of puromycin. Control cells cease proliferation and show a senescent morphology (*left* panel). Cells expressing the *p53* hairpin continue to grow (*right* panel). Photos were taken 14 d posttransfection.

A   "sense" strand

```
                                    GAA
GGUCUAAGUGGAGCCCUUCGAGUGUUA     G
CCGGGUUCACUUCGGGAGGCUCACAGU     C
UU                              GUU
```

"anti-sense" strand

B   RasV12 MEF

pShh   pShh-p53

were designed such that they contained *T7* promoter sequences at the 5' end. shRNA transcripts subjected to in vitro Dicer processing were synthesized using a Riboprobe kit (Promega). Chemically synthesized RNAs were obtained from Dharmacon, Inc.

Transfection and gene silencing assays

Cells were transfected with indicated amounts of siRNA, shRNA, and plasmid DNA using standard calcium phosphate procedures at 50%–70% confluence in 6-well plates. Dual luciferase assays (Promega) were carried out by cotransfecting cells with plasmids containing firefly luciferase under the control of the SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). Plasmids were cotransfected using a 1:1 ratio of pGL3-Control (250 ng/well) to pRL-SV40. RNAi in S2 cells was performed as previously described (Hammond et al. 2000). For stable silencing, primary MEFs (a gift from S. Lowe, Cold Spring Harbor Laboratory, NY) were cotransfected using Fugene 6 with pBabe-Ha-rasV12 and pShh-p53 (no resistance marker), according to the manufacturer's recommendations. Selection was for the presence of the activated *Ha-rasV12* plasmid, which carries a puromycin-resistance marker. The pShh-p53 plasmid was present in excess, as is standard in a cotransfection experiment. We have now generated a version of the U6 promoter vector (pSHAG-1) that is compatible with the GATEWAY system (Invitrogen), and this can be used to transport the shRNA expression cassette into a variety of recipient vectors that carry *cis*-linked selectable markers. Furthermore, we have validated delivery of shRNAs using retroviral vectors. Updated plasmid information can be obtained at http://www.cshl.org/public/science/hannon.html.

Plasmids expressing hairpin RNAs

The U6 promoter region from −265 to +1 was amplified by PCR, adding 5' *Kpn*I and 3' *Eco*RV sites for cloning into pBSSK+. A linker/terminator oligonucleotide set bearing the U6 terminator sequence and linker ends of 5' *Eco*RV and 3' *Not*I was cloned into the promoter construct, resulting in a U6 cassette with an *Eco*RV site for insertion of new sequences. This vector has been named pShh1. Blunt-ended, double-stranded DNA oligonucleotides encoding shRNAs with between 19 and 29 bases of homology to the targeted gene were ligated into the *Eco*RV site to produce expression constructs. The oligonucleotide sequence used to construct Ff1 was: TCCAATTCAGCGGGAGCCACC TGATGAAGCTTGATCGGGTGGCTCTCGCTGAGTTGGA ATCCATTTTTTTT. This sequence is preceded by the sequence GGAT, which is supplied by the vector, and contains a tract of more than five Ts as a Pol III terminator.

In vitro Dicer assays

In vitro assays for Dicer activity were performed as described (Bernstein et al. 2001a).

Acknowledgments

References

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. 2001a. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409:** 363–366.

Bernstein, E., Denli, A.M., and Hannon, G.J. 2001b. The rest is silence. *RNA* **7:** 1509–1521.

Billy, E., Brondani, V., Zhang, H., Muller, U., and Filipowicz, W. 2001. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci.* **98:** 14428–14433.

Caplen, N.J., Parrish, S., Imani, F., Fire, A., and Morgan, R.A. 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci.* **98:** 9742–9747.

Chong, S.S., Hu, P., and Hernandez, N. 2001. Reconstitution of transcription from the human U6 small nuclear RNA promoter with eight recombinant polypeptides and a partially purified RNA polymerase III complex. *J. Biol. Chem.* **276:** 20727–20734.

Clarke, P.A. and Mathews, M.B. 1995. Interactions between the double-stranded RNA binding motif and RNA: Definition of the binding site for the interferon-induced protein kinase DAI (PKR) on adenovirus VA RNA. *RNA* **1:** 7–20.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001a. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494–498.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. 2001b. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes & Dev.* **15:** 188–200.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. 2001c. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* **20:** 6877–6888.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391:** 806–811.

Gil, J. and Esteban, M. 2000. Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): Mechanism of action. *Apoptosis* **5:** 107–114.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. 2001. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106:** 23–34.

Guo, S. and Kemphues, K.J. 1995. par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81:** 611–620.

Ha, I., Wightman, B., and Ruvkun, G. 1996. A bulged lin-4/lin-14 RNA duplex is sufficient for *Caenorhabditis elegans* lin-14 temporal gradient formation. *Genes & Dev.* **10:** 3041–3050.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J.

2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404:** 293–296.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., and Hannon, G.J. 2001a. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293:** 1146–1150.

Hammond, S.M., Caudy, A.A., and Hannon, G.J. 2001b. Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet.* **2:** 110–119.

Hannon, G.J., Chubb, A., Maroney, P.A., Hannon, G., Altman, S., and Nilsen, T.W. 1991. Multiple cis-acting elements are required for RNA polymerase III transcription of the gene encoding H1 RNA, the RNA component of human RNase P. *J. Biol. Chem.* **266:** 22796–22799.

Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., and Zamore, P.D. 2001. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293:** 834–838.

Kennerdell, J.R. and Carthew, R.W. 2000. Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nat. Biotechnol.* **18:** 896–898.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes & Dev.* **15:** 2654–2659.

Knight, S.W. and Bass, B.L. 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science* **293:** 2269–2271.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. 2001. Identification of novel genes coding for small expressed RNAs. *Science* **294:** 853–858.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. 2001. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* **294:** 858–862.

Lee, R.C. and Ambros, V. 2001. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294:** 862–864.

Lobo, S.M., Ifill, S., and Hernandez, N. 1990. cis-Acting elements required for RNA polymerase II and III transcription in the human U2 and U6 snRNA promoters. *Nucleic Acids Res.* **18:** 2891–2899.

Nykanen, A., Haley, B., and Zamore, P.D. 2001. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107:** 309–321.

Paddison, P., Caudy, A.A., and Hannon, G.J. 2002. Stable suppression of gene expression in mammalian cells by RNAi. *Proc. Natl. Acad. Sci.* **99:** 1443–1448.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403:** 901–906.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88:** 593–602.

Shi, H., Djikeng, A., Mark, T., Wirtz, E., Tschudi, C., and Ullu, E. 2000. Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. *RNA* **6:** 1069–1076.

Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R., and Ruvkun, G. 2000. The lin-41 RBCC gene acts in the *C. elegans* heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol. Cell* **5:** 659–669.

Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G., and Waterhouse, P.M. 2000. Total silencing by intron-spliced hairpin RNAs. *Nature* **407:** 319–320.

Svoboda, P., Stein, P., Hayashi, H., and Schultz, R.M. 2000. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development* **127:** 4147–4156.

Tavernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A., and Driscoll, M. 2000. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nat. Genet.* **24:** 180–183.

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., and Sharp, P.A. 1999. Targeted mRNA degradation by double-stranded RNA in vitro. *Genes & Dev.* **13:** 3191–3197.

Wianny, F. and Zernicka-Goetz, M. 2000. Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.* **2:** 70–75.

Wightman, B., Ha, I., and Ruvkun, G. 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75:** 855–862.

Williams, B.R. 1997. Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem. Soc. Trans.* **25:** 509–513.

Yang, S., Tutton, S., Pierce, E., and Yoon, K. 2001. Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Mol. Cell. Biol.* **21:** 7807–7816.

RNA interference: the New Somatic Cell Genetics?

Patrick J. Paddison & Gregory J. Hannon*

Cold Spring Harbor Laboratory
Watson School of Biological Sciences
1 Bungtown Road
Cold Spring Harbor, NY

*To Whom Correspondence Should Be Addressed:
Gregory J. Hannon
Phone : 516-367-8889
Fax : 516-367-8874

Since the 1970s, the war on cancer has been based on the notion that studying the disease will lead to the discovery of vulnerabilities, which can be exploited in the clinic. While many underlying genetic determinants of cancer have been identified, this knowledge has failed to translate into new therapeutic strategies, with only a handful of exceptions. One hypothesis is that this failure has largely been due to the genetically intractable nature of cultured mammalian cells. The recent emergence of dsRNA-induced gene silencing, or RNA interference (RNAi), in mammalian systems is likely to re-invigorate the field of somatic cell genetics and in the process revolutionize the study of human disease. During the past year, a point has been reached at which any gene in the human genome can conceivably be targeted using small dsRNA gene silencing triggers – *small interfering RNAs (siRNAs) or expressed short hairpin RNAs (shRNAs)*. The application of siRNAs and shRNAs for single gene analysis is rapidly becoming standard methodology, and genome-wide reverse genetic screens are certainly on the horizon. The future of RNAi may lie in the development of highly specific, nucleic-acid based therapies for cancer and other diseases.

RNAi in invertebrate systems

RNAi first emerged as a biological oddity in *C. elegans* and plants but was quickly harnessed into to a powerful genetic tool in these systems. However, it has become clear that dsRNA-induced silencing phenomena are present in evolutionarily diverse organisms including plants, fungi, and metazoans (reviewed in Bernstein et al., 2001b; Hammond et al., 2001b). A combination of genetic and biochemical studies suggest that many of these phenomena share a common mechanism. The prevailing model begins with the conversion of the dsRNA silencing "trigger" into small RNAs (siRNAs) by an RNAse III family nuclease, Dicer (Bernstein et al., 2001). These small RNAs (~22-25nt in size)

become incorporated into a multicomponent nuclease complex, which uses the sequence of the siRNAs as a guide to identify and destroy homologous mRNAs (Hammond et al., 2000; Zamore et al., 2000).

So far the only universally conserved players in RNAi are Dicer and Argonaute (Ago) gene family members. Dicer contains a tandem repeat of RNaseIII catalytic domains, a carboxyl-terminal dsRNA-binding domain, an amino-terminal DExH/DEAH RNA helicase domain, and a PAZ domain (Berstein et al., 2001; Nicholson and Nicholson, 2002). Ago proteins, which are required for the formation of the RNA-induced silencing complex (RISC), contain a PAZ domain and a carboxyl-terminal PIWI domain.

The RNAi pathway may have evolved early in eukaryotes as a cell-based immunity against viral and genetic parasites. Double-stranded RNA viruses or genomic retroelements with dsRNA intermediates are ubiquitously found in nature and are sometimes subject to RNAi-dependent gene silencing in *C. elegans*, plants, Drosophila, yeast, and mammals (reviewed in Hannon, 2002). However, the RNAi pathway is also used for the regulation of endogenous gene targets during metazoan and plant development (reviewed in Hannon, 2002).

Endogenously expressed small hairpin RNAs regulate the expression of developmental genes through the RNAi pathway during *C. elegans* development (Reinhart et al., 2000; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; reviewed in Hannon, 2002). These small hairpin RNAs (~70nt) are processed into a 21-22nt mature form by Dicer and then used to seek out mRNA targets of similar sequence (generally via imperfect base-pairing interactions). For the two prototypes of this family, *C. elegans* lin-4 and let-7, silencing occurs at the level of protein synthesis (reviewed in Hannon, 2002). The first small hairpin RNAs were dubbed small temporal RNAs (stRNAs), owing to their role in developmental timing (Lee et al., 1993; Ha et al, 1996). More recently, dozens of orphan hairpins have been identified in *C. elegans*, mouse, and humans, which

are collectively referred to as microRNAs (miRNAs) (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; *).

RNAi underlies many homology-dependent silencing phenomena in invertebrates: cosuppression, virus-induced gene silencing, transgene-induced silencing, and quelling (reviewed in Bernstein et al., 2001b). These silencing phenonema variably involve either Post-Transciriptional Gene Silencing (PTGS), Transcriptional Gene Silencing (TGS) or both. RNAi in *C. elegans* appears to solely involve PTGS, while in plants the same dsRNA trigger can target both mRNA and chromatin.

RNAi in mammals

Given the strong conservation of RNAi-related genes in vertebrates, including Dicer and Argonaute family members, the expectation was that RNAi would operate mammalian cells in some capacity. The first glimpse of RNAi in mammals came from injections of long dsRNAs (~500 nt, similar to those used to trigger RNAi in invertebrate systems) into mouse embryos, which resulted in sequence-specific gene silencing (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000). Several groups, including our own, extended these findings to embryonal cell lines (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002a). Biochemical and genetic evidence from these studies suggested that RNAi operates in at least a subset of mammalian cell types, in a Dicer-dependent manner via post-transcriptional mechanisms (Billy et al., 2001; Paddison et al., 2002a).

In somatic cells, however, the use of conventional dsRNA triggers (~500nt dsRNAs) is limited by antiviral/ interferon responses, including the PKR and RNaseL pathways (Baglioni and Nilsen, 1983; reviewed in Williams, 1997), which trigger translational repression and apoptosis in response to dsRNA of >30bp in length. Even where PKR activity is removed from somatic cells, either

by viral inhibitors or targeted disruption, long dsRNA still triggers a residual non-specific repression of gene expression (Abraham et al., 1999; Paddison et al., 2002a; P. Paddison and G. Hannon, unpublished results).

One way around these non-specific dsRNA responses is to simply create dsRNA triggers of RNAi <30bp in length. In the past year, two short dsRNA structures have emerged, which evoke sequence specific gene silencing in somatic cells without activating anti-viral responses. These are the small interfering RNA (siRNA) and the short hairpin RNA (shRNA). Both are modeled after biologically active structures in the RNAi pathway: Dicer cleavage products and small temporal RNAs, respectively.

Tuschl and colleagues (Elbashir et al., 2001) first demonstrated that small dsRNAs of ~21 nt, resembling siRNAs from other systems, can induce sequence specific gene silencing when transiently transfected into mammalian cells. These small interfering RNAs (siRNAs) are chemically synthesized emulations of Dicer cleavage products, which are short RNA duplexes ~19 nt in length with 2 nt 3' overhangs on each strand. The siRNAs presumably bypass the requirement for Dicer and enter the silencing pathway by incorporation into RISC complexes. The use of siRNAs has been recently reviewed, in detail, and resources for the design and use of siRNAs are available from Tom Tuschl's laboratory online (www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html).

As an alternative strategy, we and others have developed *in vivo* expression constructs for small dsRNA triggers in mammalian cells, which resemble endogenously expressed hairpin RNAs (Paddison et al., 2002b; Brummelkamp et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Zeng et al., 2002). This approach uses small inverted repeats (19-29nt) expressed from RNA polymerase III promoter to create short hairpin RNAs (shRNAs), which can then be processed by Dicer and shunted into the RNAi pathway. However, siRNAs can also be produced in vivo by the expression of

complementary 19 or 21 nt RNAs from separate RNA polymerase III transcription units (Lee et al., 2002; Myagishi and Taira, 2002; Yu et al., 2002).

All of the forgoing methods for generating silencing triggers *in vivo* have been applied to knocking down endogenous genes. Expressed dsRNA triggers have a potential advantage when combined with well-worn strategies for stable and inducible gene silencing *in vitro* and *in vivo*. The details of these strategies are further discussed below.

One of the major differences between mammalian cell RNAi and the response observed, for example, in *C. elegans* is the apparent lack of amplification of the RNAi effect. In *C. elegans,* "amplification" results in heritable, systemic gene silencing. According to one model, amplification of the dsRNA signal is initially mediated by RNA-dependent RNA polymerases (RdRP). Guide RNAs (i.e., siRNAs) serve to prime RdRPs along the mRNA template, resulting in the production of dsRNA of sequences 5' (i.e., upstream) to that of the targeted sequence (*). When combined with transport, amplification results in a self-propagating silencing effect throughout the organism. In mammalian cell systems, however, transient transfection of RNAi triggers, e.g., long dsRNA, siRNAs or shRNAs, results in a transient effect, lasting 2-7 days. The longevity of silencing is likely dependent on gene expression homeostasis (e.g., abundance of mRNA and protein, stability of the protein, transcriptional feedback loops, etc.), the half-life of the silencing complex itself, and cell division, which serves to dilute the effect over time.

Design and Expression of dsRNA triggers.

Tuschl and colleagues have elaborated several guidlines for designing siRNA oligos for chemical synthesis (www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html; Elbashir et al., 2002). The selection of the target sequence should avoid regions of the mRNA which

might bind RNA regulatory proteins, such as 5' and 3' UTR and regions close to the the start site (<100nt). Between +100nt (with the AUG referenced as +1) and the stop codon, 23nt sequences resembling 5'-AA(N19)UU-3', where N is any nucleotide, are selected in the mRNA sequence. Sequences of >70% or <30% GC content or which are highly G-rich should be avoided. The siRNA is then constructed by designing sense and anti-sense (i.e., reverse complement) N19 sequences, each ending with two 3' 2-deoxythymidine residues. Refer to Elbashir et al (2002) for a more detailed protocol. The current average cost for chemical synthesis siRNAs is between $270 to $500 per siRNA depending on purification and scale of synthesis.

Two less costly strategies for generating siRNAs involve *in vitro* transcription reactions using T7 polymerase (Paddison et al., 2002b, Yu et al., 2002; Donze and Picard, 2002; with commercial kits for this purpose available from New England Biolabs and Ambion) and *in vitro* processing of long dsRNA using the Dicer enzyme (J. Myers and J. Ferrell, unpublished results). T7 generated siRNAs differ from normal Dicer products in that they contain 5'-triphosphates. Despite this difference T7-siRNAs have been shown to be biologically active. A T7-siRNA primer design program and detailed instructions is available at (www.cshl.org/public/SCIENCE/hannon.html) *In vitro* processing of long dsRNA by purified Dicer enzyme may eventually be the most effective way to generate siRNAs, since the end-products will be a mixture of dozens of separate siRNAs targeting a single mRNA. However, siRNA populations will likely require purification to avoid contamination of long dsRNA, and the siRNA populations may have a higher probability of targeting other genes than do discrete siRNAs. Regardless of the method used to generate siRNAs, the major drawback of exogenously produced siRNAs is the inability to stably or inducibly regulate gene expression.

Toward this end, we and others have developed expression strategies for dsRNA triggers in embryonal cell types (Billy et al., 2001; Yang et al., 2001;

Paddison et al., 2002a) and in somatic cells (Brummelkamp et al., 2002; Lee et al., 2002; Myagishi and Taira, 2002; Paddison et al., 2002b; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002; Zeng et al., 2002). Three types of expressed dsRNA triggers have been used with apparent success for stable or transient gene silencing: long dsRNA hairpins, short hairpin RNAs (shRNAs), and co-expressed small sense and anti-sense RNAs (i.e., that anneal to form siRNAs).

For embryonal cell types in which the PKR/interferon response is diminished or absent, long dsRNAs can be formed by expression of long inverted repeats (*). In undifferentiated cells, clones can be obtained, which stably suppress gene expression at a frequency similar to that observed for expression of transgenes via plasmid integration (~30%) (Paddison et al., 2002a). However, the utility of long dsRNA is likely limited to undifferentiated stem cells or EC cells since upon differentiation silencing is lost for unknown reasons (Wianny and Zernicka-Goetz, 2000; Yang et al., 2001).

For cells derived from somatic tissues, a flurry of recent reports demonstrates that expression of short hairpin RNAs (shRNAs) or complementary siRNA strands leads to sequence specific gene silencing. The basic expression schemes and expression strategies are presented in Figure *. Most strategies use RNA polymerase III promoters (either human or mouse U6-snRNA or human RNase P (H1) RNA promoters) to drive expression of short RNAs, since RNA polymerase III can be exploited to precisely initiate and terminate RNA transcripts (Goomer and Kunkel, 1992). These promoters should be active in most if not all embryonal and somatic cell types. One group, however, successfully triggered silencing by burying a microRNA structure within a RNA polymerase II derived transcript (Zeng et al., 2002).

There are a few points to note concerning the expression strategies that have been used thus far. First, U6 derived shRNAs and siRNAs have sequence constraints, where a G residue is required for efficient initiation (Goomer and

Kunkel, 1992***?). Second, one report suggests that adding the leader sequence of 27 nt from the U6 snRNA improves expression (Paul et al., 2002). Third, there is *in vitro* data to suggest that the mouse U6 promoter may be more active in human cells, given the strong affinity of the SNAP-c complex for the proximal sequence element (Chong et al., 2001 ****?). However, given the published data, and our unpublished results, the minor differences amongst the reported expression strategies are unlikely to have a major impact on the efficacy of silencing.

Surprisingly, many of the structural features present in micro RNAs can be ignored in shRNAs expressed from RNA polymerase III promoters (*). Such features include loop and stem structure and length of the stem (19-29nt). When using the human U6 and H1 promoters, we have found that loop structures of 4 or 8 nt work equally well when comparing the same 19 nt and 29 nt stems (unpublished results). Structured stems and loops, modeled after human let-7-like hairpins (Pesquinelli et al., 2000; Lagos-Quintana et al., 2001), work considerably less well than perfectly matched stems with simple loops (Paddison et al., 2002b; P. Paddison, D. Conklin, and G. Hannon, unpublished data). The only differences in efficiency, which arise, appear to be dependent upon length of the hairpin stem, where stems of 29nt work 10-40% more efficiently than stems of 19nt, at least when targeting reporter genes (P. Paddison, D. Conklin, and G. Hannon, unpublished results). This modest increase in efficiency must, however, be balanced against concerns that longer stems could theoretically increase the possibility of off-target effects. Thus, the optimal structure for shRNAs will likely emerge only after the accumulation of copious additional *in vivo* data with large numbers of genes.

Whichever method is used, we suggest selecting 3-6 shRNA sequences per gene. Empirical data suggests that one or more RNAi expression constructs should give 40-90% reduction in gene expression when used transiently. A

program for constructing shRNA (29nt stems) cloning primers, along with detailed protocols, are available at www.cshl.org/public/SCIENCE/hannon.html.

By default, we use rules similar to those described by Tuschl and colleagues for choosing shRNA targeting sequences. A target sequence of 5'-(N28)C-3', where N is any nucleotide and N28 contains between 30-70% GC, is selected from the target mRNA. We engineer the 5' stem strand as the "anti-sense" strand; however, either strand is effective (P. Paddison, D. Conklin, and G. Hannon, unpublished data ** can we reference all the others here **). To aid in cloning and to increase stability in bacteria, we incorporate G-U base pairs in the stem of shRNAs, which are permitted in duplexed RNA but not DNA. However, this strategy has not been used by others and is likely non-essential (*). Although we have no direct evidence regarding G-U base pairs and target mRNA recognition, changes are made in only the "sense" stem strand (Paddison et al., 2002b).

Delivery Strategies

Both siRNAs and vectors containing dsRNA triggers can be transiently transfected into mammalian cells using any commonly available transfection reagent. Chemically synthesized siRNAs are effective at concentrations ranging from * to * in transient transfections, while U6-shRNA vectors are effective at concentrations normally used for expression of transgenes.

There are a number of well-characterized stable expression technologies currently being used in mammalian cells, which should permit permanent expression of shRNA and siRNA in target cells (Fig *). These include systems based on retroviral integration (e.g., Hannon et al., 1999; Lois et al., 2002), transposon hopping (e.g., Ivics et al., 1997), episomally replicated DNA fragments (e.g., Chittenden et al., 1989; Sedman and Stenlund, 1995), and homologous recombination (e.g., Nagy, 2000).

Among recent reports, stable RNAi has been demonstrated using random plasmid integration (Brummelkamp et al., 2002; Paddison et al., 2002b) and episomal plasmid maintenance (Myagishi and Taira, 2002). However, based upon our observations, stable maintenance of RNAi following plasmid integration may be problematic where the RNAi effect itself is not positively selected (e.g., bypass of senescence). Therefore, we have begun exploring retroviral strategies for stable expression of shRNAs. We have found that cells transduced MoMuLV or MSCV vectors harboring U6-shRNA cassettes can stably evoke RNAi. Fig * shows bypass of rasV12 induced senescence in early passage mouse embryo fibroblasts using a mouse p53 shRNA (Paddison et al., 2002b) expressed from pBABE-puro (*). MEFs co-transduced with Wzl-rasV12 and Babe-puro alone show a flattened morphology and growth arrest consistent with cellular senescence (Ferbeyre et al., 2000 * cite serrano), while cells co-transduced with Wzl-rasV12 and Babe-puro-U6-shRNA-p53 display a transformed morphology with little or no observable growth arrest. With retrovirus-based strategies, the expression of shRNA during virus packaging may result in reduced virus production. For example, shRNAs may target the shRNA sequences contained on viral transcripts or the markers used for selecting infected cells. Furthermore, targeting essential genes is likely to have adverse effects on packaging cells. The movement to inducible RNA pol III promoter should ameliorate these problems, and we have recently derived an activator dependent, U6-based expression system for shRNAs (P. Paddison, E. Julien, W. Herr and G. Hannon, unpublished)

The forgoing, preliminary results suggest that retroviral vectors may represent potent delivery strategies for shRNA expression. Of particular interest is the potential to create transgenic animals through the transduction of preimplantation embryos or ES cells with lentiviral vectors (Lois et al., 2002; Pfeifer et al., 2002).

RNAi-based screens: applications in cancer cells.

In other model systems, the use of genetic screens to explore functional dependencies has been an enabling feature of countless discoveries. For example, analysis of temperature sensitive mutants in bacteriophage T4 lead to the discovery of viral morphogenesis modules (Edgar and Wood, 1966). Similar approaches in yeast revealed functional hierarchies among genes regulating cell cycle progression (Hartwell et al., 1974; Hartwell and Weinert, 1989). The key to such discoveries has been the ability to create recessive, genetically defined lesions in molecular pathways.

Since cancer arises from genetic lesions in somatic cells, the concept of synthetic lethality has been heralded as way to functionally define vulnerabilities in cancer cells (Hartwell et al., 1997). Synthetic lethal interactions occur when mutations in two or more non-allelic genes synergize to kill cells. For example, a mutation in gene A or gene B may be tolerated when singly present in cells, but when combined result in a loss of viability. Thus, synthetic lethal interactions reveal situations in which cellular homeostasis is altered by a molecular lesion so that the action of another gene or pathway is required to compensate. The fact that cancer cells arise from genetic alterations makes synthetic lethality ideally suited for identifying cellular targets required by cancer cells for viability.

Our group is currently in the process of undertaking large-scale RNAi-based screens for lethal targets in cancer cell lines. The biggest question in regard to designing mammalian cell screens is whether to use forward or reverse genetic approaches. Randomized, forward genetic screens have been used with some degree of success in mammalian systems for gain of function genetic lesions. Such screens generally consist of expressing, in mass, cDNAs or genomic fragments in receipt cell populations and screening for a positively selectable phenotype (e.g., Deiss and Kimchi, 1991; Wong et al., 1994; Maestro et al., 1999 ** roninson, gudkov). Perhaps the best example of this type of

approach came early on with the cloning of the ras oncogene from genomic libraries in rodent cells (Goldfarb et al., 1982; Shih and Weinberg, 1982). While such approaches are compatible with RNAi-induced phenotypes (e.g., bypass of senescence – Paddison et al, 2002b), a well-to-well, reverse genetic approach has two major advantages. First, neutral or negatively selected phenotypes (e.g., apoptosis, growth arrest) can be scored in each well for single and multiple gene targeting events. Second, RNAi expression constructs can be assembled into restricted functional sets *a priori* based on known or inferred function of gene targets (e.g., DNA replication, DNA damage repair, etc.). Comparing phenotypic readouts among different restricted sets may give rise to "epistasis signatures", or maps of functional dependencies underlying a particular phenotype in a particular genetic background (e.g., transformed versus non-transformed cells). Such signatures would be comparable to transcript array patterns, except that epistasis signatures would be functionally defined and thus more suggestive of cause and consequence.

Perspectives.

Through the use of reverse genetic approaches, RNAi has developed into a powerful tool for probing gene function in *C. elegans* and other invertebrate systems. In worms, RNAi is currently being used to systematically target ~19,000 predicted genes (J. Ahringer, personal communication). Similar approaches are underway in plants (D. Baulcombe and P. Waterhouse, personal communication). With the added capacity of RNAi, somatic mammalian cells will hopefully gain admittance into the pantheon of model genetic systems. In practical terms, the use of RNAi in cultured cells may deliver new insights into a host of disease related processes, including concrete information on drug targets.

RNAi also holds promise for *in vivo* genetic applications in mammals. Perhaps the most immediate question is whether expressed RNAi triggers can

be combined with transgenic approaches for stably knocking down gene expression in rodents. *Ex vivo* cell implantation studies can also benefit from RNAi, where primary or transformed cells are stably engineered with shRNAs and then implanted into mice. Inducible RNAi triggers may ultimately key components of both *in vivo* and *ex vivo* approaches in rodent systems.

In humans, there are many scenarios, which can be envisioned where RNAi could be enlisted to combat disease. These include targeting viral pathogens, targeting disease- or symptom-causing genes (or alleles), modifying primary cells, *ex vivo,* to remove undesirable gene products, expressing shRNAs from replication competent viruses to selectively kill cancer cells, and so on. With regard to target specificity, dsRNA triggers of gene silencing represent ideal therapeutic molecules, since gene products are targeted based on mRNA sequence rather than protein activity, and are thus not limited by the ability of medicinal chemistry to target a protein class or interaction. However, at present, effective delivery strategies present a significant barrier to therapeutic applications of RNAi.

RNAi shows tremendous promise as a new technology for manipulating gene expression for both experimental and therapeutic purposes. However, we are still in the very early stages of understanding both the mechanistic basis and biological roles of these gene-silencing pathways. Thus, we will undoubtedly see both spectacular successes and notable failures of RNAi before we fully understand the power and limitations of this new tool.

References.

Abraham N., Stojdl D.F., Duncan P.I., Methot N., Ishii T., Dube M., Vanderhyden B.C., Atkins H.L., Gray D.A., McBurney M.W., Koromilas A.E., Brown E.G., Sonenberg N. and Bell J.C. (1999) Characterization of transgenic mice with targeted disruption of the catalytic domain of the double-stranded RNA-dependent protein kinase, PKR. *J. Biol. Chem.*, 274:5953-62.

Avner P. and Heard E. (2001) X-chromosome inactivation: counting, choice and initiation. *Nat. Rev. Genet.*, 2:59-67.

Baglioni C. and Nilsen T. W. (1983) Mechanisms of antiviral action of interferon. *Interferon*, 5:23-42.

Bernstein E., Caudy A.A., Hammond S.M. and Hannon G.J. (2001a) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409:363-6.

Bernstein E., Denli A.M. and Hannon G.J. (2001b) The rest is silence. *Rna*, 7:1509-21.

Billy E., Brondani V., Zhang H., Muller U. and W. Filipowicz W. (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci.*, 98: 14428-33.

Brummelkamp T.R., Bernards R. and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, 296: 550-3.

Caplen N.J.,Parrish S., Imani F., Fire A. and Morgan R.A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci.*,98: 9742-7.

Chittenden T., Lupton S., Levine A.J. (1989) Functional limits of oriP, the

Epstein-Barr virus plasmid origin of replication. *J. Virol.*, 63:3016-25.

Chong S.S., Hu P., and Hernandez N. (2001) Reconstitution of transcription from the human U6 small nuclear RNA promoter with eight recombinant polypeptides and a partially purified RNA polymerase III complex. *J. Biol. Chem.*, 276:20727-34.

Clarke P.A. and Mathews M.B. (1995) Interactions between the double-stranded RNA binding motif and RNA: definition of the binding site for the interferon-induced protein kinase DAI (PKR) on adenovirus VA RNA. *Rna*, 1: 7-20.

Deiss L.P. and Kimchi A. (1991) A genetic tool used to identify thioredoxin as a mediator of a growth inhibitory signal. *Science*, 252117-20.

Donze O. and Picard D. (2002) RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase.*Nucleic Acids Res.*, 30:e46.

Edgar R. and Wood W. (1966) Morphogenesis of bacteriophage T4 in extracts of mutant-infected cells. *Proc. Natl. Acad. Sci.*, 55: 498-505.

Elbashir S.M., Harborth J., Lendeckel W., Yalcin A., Weber K. and Tuschl T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494-8.

Elbashir S.M., Harborth J., Weber K. and Tuschl T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods*, 26:199-213.

Ferbeyre G., de Stanchina E., Querido E., Baptiste N., Prives C. and Lowe S.W. (2000) PML is induced by oncogenic ras and promotes premature senescence. *Genes Dev.*, 14:2015-27.

Fire A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E. and Mello C.C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391:806-11.

Gil J. and Esteban M. (2000) Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis*, 5:107-14.

Grishok A., Pasquinelli A.E., Conte D., Li N., Parrish S., Ha I., Baillie D.L., Fire A., Ruvkun G. and Mello C.C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* 106: 23-34.

Goldfarb M., Shimizu K., Perucho M. and Wigler M. (1982) Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. *Nature*, 296:404-9.

Goomer R.S. and Kunkel G.R. (1992) The transcriptional start site for a human U6 small nuclear RNA gene is dictated by a compound promoter element consisting of the PSE and the TATA box. *Nucleic Acids Res.*, 20:4903-12.

Guo S. and Kemphues K.J. (1995) par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell*, 81: 611-20.

Ha I., Wightman B. and Ruvkun G. (1996) A bulged lin-4/lin-14 RNA duplex is sufficient for Caenorhabditis elegans lin-14 temporal gradient formation. *Genes Dev.*, 10:3041-50.

Hammond S.M., Bernstein E., Beach D. and Hannon G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, 404:293-6.

Hammond S.M., Boettcher S., Caudy A.A., Kobayashi R. and Hannon G.J. (2001a) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science*, 293:1146-50.

Hammond S.M., Caudy A.A. and Hannon G.J. (2001b) Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet.*, 2:110-9.

Hannon G.J. (2002) RNAi. *Nature*, in press.

Hartwell L.H., Culotti J., Pringle J.R. and Reid B.J. (1974) Genetic control of the cell division cycle in yeast. *Science*, 183:46-51.

Hartwell L..H. and Weinert T. A. (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science*, 246:629-34.

Hartwell L.H., Szankasi P., Roberts C.J., Murray A.W. and Friend S.H. (1997)

Integrating genetic approaches into the discovery of anticancer drugs. *Science*, 278:1064-8.

Hutvagner G., McLachlan J., Pasquinelli A.E., Balint E., Tuschl T. and Zamore P.D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science*, 293:834-8.

Ivics Z., Hackett P.B., Plasterk R.H. and Izsvak Z. (1997) Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. *Cell*, 91:501-10.

Ketting R.F., Fischer S.E., Bernstein E., Sijen T., Hannon G.J. and Plasterk R.H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev.*, 15: 2654-9.

Knight S.W. and Bass B.L. (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science*, 293: 2269-71.

Lagos-Quintana M., Rauhut R., Lendeckel W.and Tuschl T. 2001. Identification of novel genes coding for small expressed RNAs. *Science*, 294:853-8.

Lau N.C., Lim L.P., Weinstein E.G., and Bartel D.P. (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science*, 294:858-62.

Lee N.S., Dohjima T., Bauer G., Li H., Li M.J., Ehsani A., Salvaterra P. and Rossi J. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol.*, 20:500-5.

Lee R.C., Feinbaum R.L. and Ambros V. (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75:843-854

Lee R.C. and Ambros V. (2001) An extensive class of small RNAs in Caenorhabditis elegans. *Science*, 294:862-4.

Lois C., Hong E.J., Pease S., Brown E.J. and Baltimore D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science*, 295:868-72.

Maestro R., Dei Tos A.P., Hamamori Y., Krasnokutsky S., Sartorelli V., Kedes L., Doglioni C., Beach D.H. and Hannon G.J. (1999) Twist is a potential oncogene that inhibits apoptosis. *Genes Dev.*, 13:2207-17.

Miyagishi M. and Taira K. (2002) U6 promoter driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.*, 20:497-500.

Nagy A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis*, 26:99-109

Nicholson R.H. and Nicholson A.W. (2002) Molecular characterization of a mouse cDNA encoding Dicer, a ribonuclease III ortholog involved in RNA interference. *Mamm Genome*,13:67-73.

Nykanen A., Haley B., and Zamore P.D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*, 107: 309-21.

Paddison P.J., Caudy A.A. and Hannon G.J. (2002a) Stable Suppression of Gene Expression in Mammalian Cells by RNAi. *Proc. Natl. Acad. Sci.*, 99:1443-1448.

Paddison P.J., Caudy A.A., Bernstein E., Hannon G.J. and Conklin D.S. (2002b) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.*, 16:948-58.

Paul C.P., Good P.D., Winer I. and Engelke D.R. (2002) Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.*, 20: 505-8.

Pasquinelli A.E., Reinhart B.J., Slack F., Martindale M.Q., Kuroda M.I., Maller B., Hayward D.C., Ball E.E., Degnan B., Muller P., Spring J., Srinivasan A., Fishman M., Finnerty J., Corbo J., Levine M., Leahy P., Davidson E. and Ruvkun G. (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature*, 408:86-9.

Pfeifer A., Ikawa M., Dayn Y. and Verma I.M. (2002) Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc. Natl. Acad. Sci.*, 99:2140-5.

Pruitt K.D. and Maglott D.R. (2001) RefSeq and LocusLink: NCBI gene-centered

resources. *Nucleic Acids Res.*, 29: 137-40.

Reinhart B.J., Slack F.J., Basson M., Pasquinelli A.E., Bettinger J.C., Rougvie
A.E., Horvitz H.R., and Ruvkun G. 2000. The 21-nucleotide let-7 RNA
regulates developmental timing in Caenorhabditis elegans. *Nature*,
403:901-6.

Sedman J. and Stenlund A. (1995) Co-operative interaction between the initiator
E1 and the transcriptional activator E2 is required for replicator specific
DNA replication of bovine papillomavirus in vivo and in vitro. *EMBO
J.*,14:6218-28.

Shi H., Djikeng A., Mark T., Wirtz E., Tschudi C.and Ullu E. (2000) Genetic
interference in Trypanosoma brucei by heritable and inducible double-
stranded RNA. *Rna*, 6:1069-76.

Shih C. and Weinberg R.A. (1982) Isolation of a transforming sequence from a
human bladder carcinoma cell line. *Cell*, 29:161-9.

Slack F.J., Basson M., Liu Z., Ambros V., Horvitz H.R. and G. Ruvkun G. (2000)
The lin-41 RBCC gene acts in the C. elegans heterochronic pathway
between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol.
Cell*, 5: 659-69.

Smith N.A., Singh S.P., Wang M.B., Stoutjesdijk P.A., Green A.G. and
Waterhouse P.M. (2000) Total silencing by intron-spliced hairpin RNAs.
Nature, 407:319-20.

Svoboda P., Stein P., Hayashi H. and Schultz R.M. (2000) Selective reduction of
dormant maternal mRNAs in mouse oocytes by RNA interference.
Development, 127:4147-56.

Tavernarakis N., Wang S.L., Dorovkov M., Ryazanov A. and Driscoll M. (2000)
Heritable and inducible genetic interference by double-stranded RNA
encoded by transgenes. *Nat. Genet.*, 24:180-3.

Tuschl T., Zamore P.D., Lehmann R., Bartel D.P. and Sharp P.A. (1999)
Targeted mRNA degradation by double-stranded RNA in vitro. *Genes
Dev.*, 13:3191-7.

Wianny F. and Zernicka-Goetz M. (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell. Biol.*, 2: 70-5.

Wightman B., Ha I. and Ruvkun G.(1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*, 75: 855-62.

Williams B.R. (1997) Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem. Soc. Trans.*, 25: 509-13.

Wong B.Y., Chen H., Chung S.W. and Wong P.M. (1994) High-efficiency identification of genes by functional analysis from a retroviral cDNA expression library. *J Virol.*, 68:5523-31.

Yang S., Tutton S., Pierce E. and Yoon K. (2001) Specific double-stranded RNA interference in undifferentiated mouse embryonic stem cells. *Mol. Cell. Biol.* 21: 7807-16.

Yu J.Y., DeRuiter S.L. and Turner D.L. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci.*, 99:6047-52.

Zamore P.D., Tuschl T., Sharp P.A. and Bartel D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, 101:25-33.

Zeng Y., Wagner E.J. and Cullen B.R. (2002) Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Molec. Cell*, in press*.
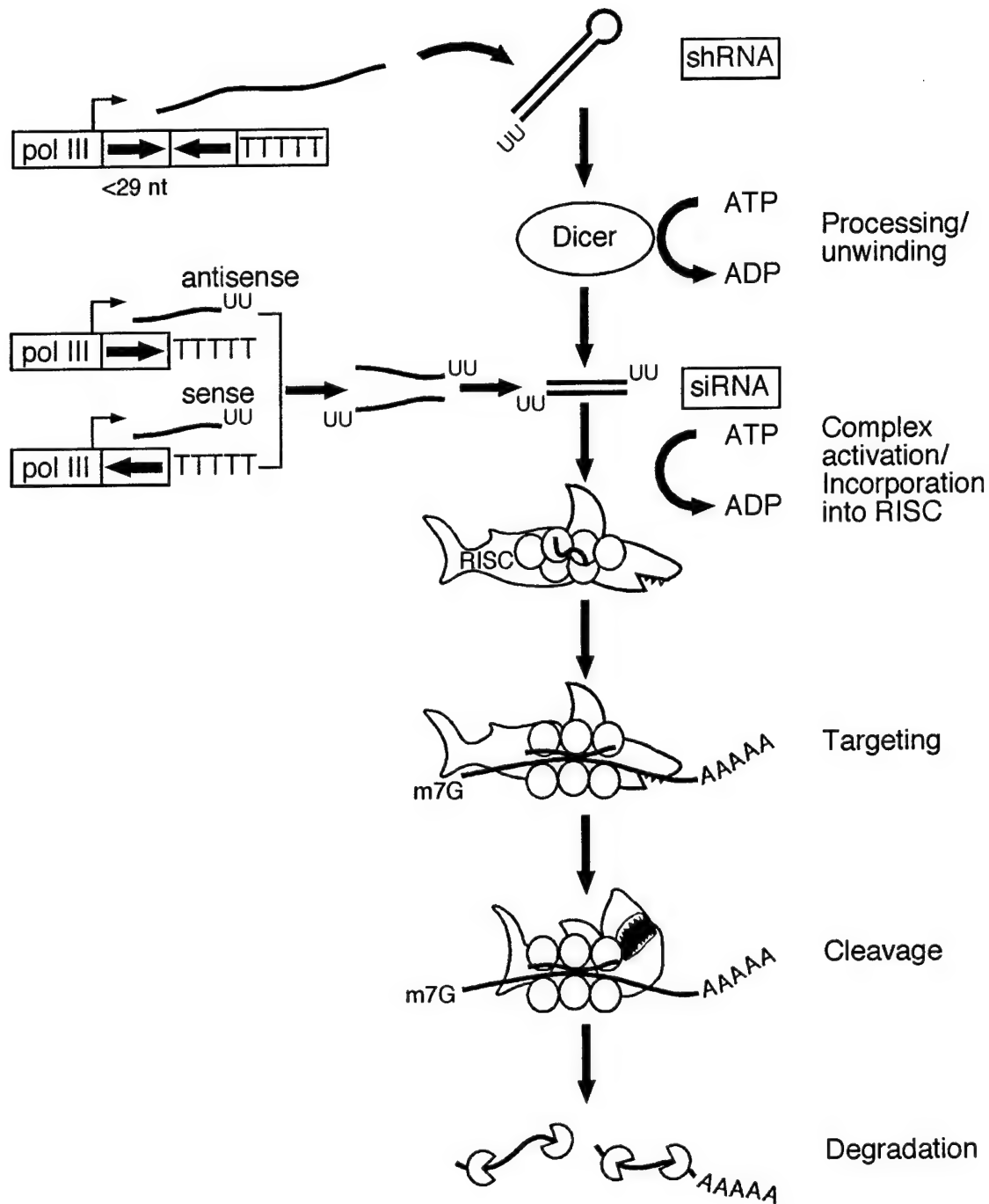
Figure Legends.

Figure 1: A model for RNA interference in mammalian cells. Small double-stranded RNA triggers of RNAi (shRNAs and siRNAs) are shown, expressed from RNA polymerase III promoters. Short hairpin RNAs (shRNAs), containing 19-29nt dsRNA stems, are processed by Dicer and incorporated into the RNA induced silencing complex (RISC), resulting in the targeting and degradation of cognate mRNAs. Small interfering RNAs (siRNAs), containing 19nt or 21nt of dsRNA, presumably bypass the requirement for Dicer and are directly incorporated into RISC.
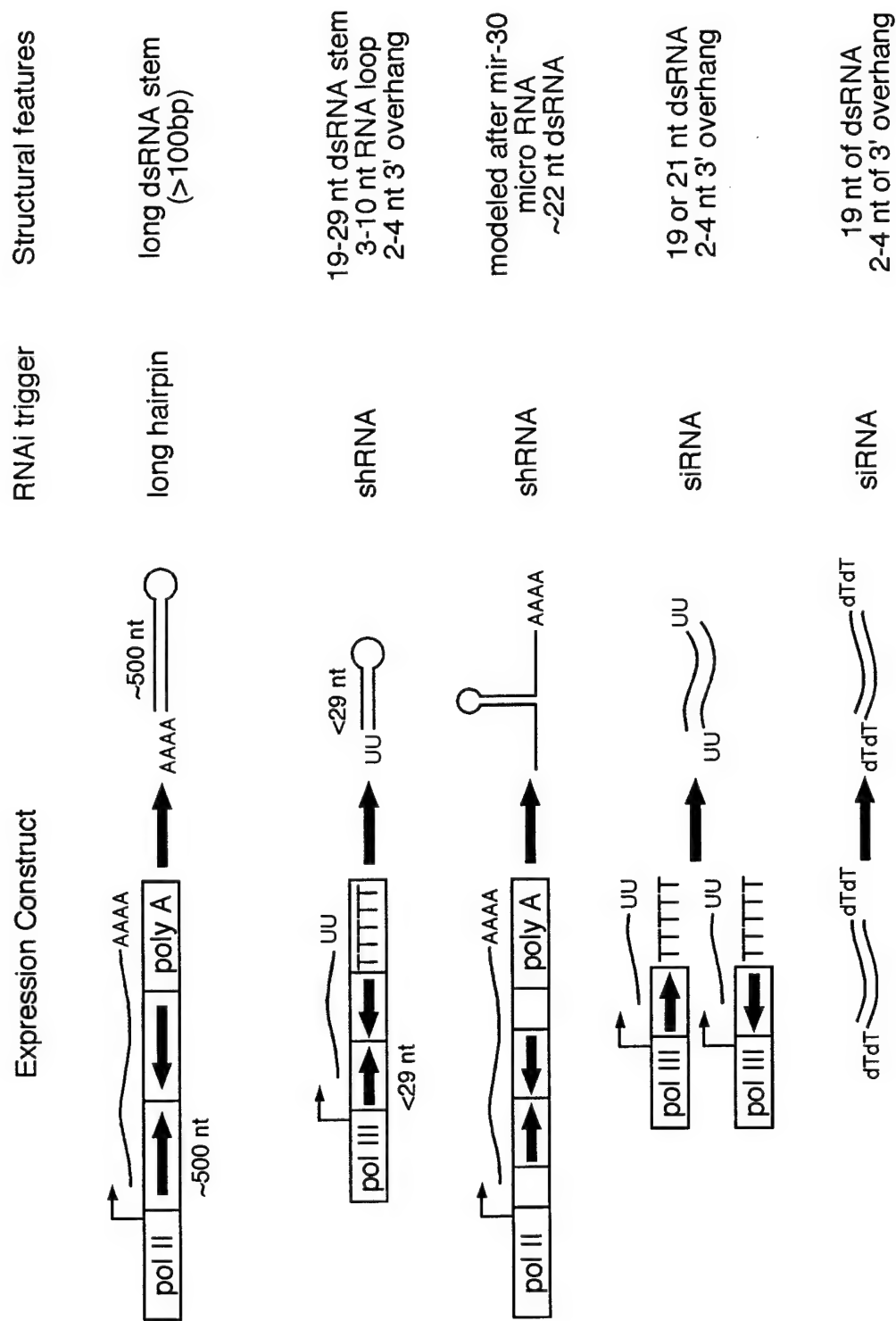
Figure 2: Expression of dsRNA triggers in mammalian cells. This figure shows the various strategies, which have been used to express dsRNA triggers in mammalian cells and some of the structural features of the dsRNA triggers. Of note is that long hairpins have so far only proven effective in embryonal cell types, which lack PKR/interferon responses. Expressed shRNAs and siRNAs, however, evoke sequence specific silencing in numerous cell types tested.

Figure 3: Stable expression of an shRNA using a retroviral system. This figure demonstrates one strategy for expressing shRNAs from retroviruses. A U6-p53-shRNA was inserted into the 3' LTR of a MoMuLV pBabe-Puro retroviral construct. **A.** Shows the predicted structure and orientation of the retrovirus as integrated into the genome of the infected cell. **B.** The predicted structure of a murine p53 shRNA. **C.** An assay for bypass of rasV12 induced senescence in early passage mouse embryo fibroblasts (P2). Cells were first transduced with Babe-Puro alone or Babe-Puro-LTR-U6-p53-shRNA and selected in puromycin for 3 days, after which cells were infected with Wzl-Hygro-rasV12 and treated with hygromycin. Only cells initially receiving Babe-Puro-LTR-p53-shRNA were morphologically transformed by rasV12 and continued to divide (see text). A time point 5 days after transduction with rasV12 is shown. Potential complications with this strategy would arise if shRNAs target viral and drug

resistance transcripts in packaging or target cells, or if shRNAs target essential genes in the packaging cells. Thereby we are currently designing inducible U6 constructs for expression from self-inactivating retroviruses.
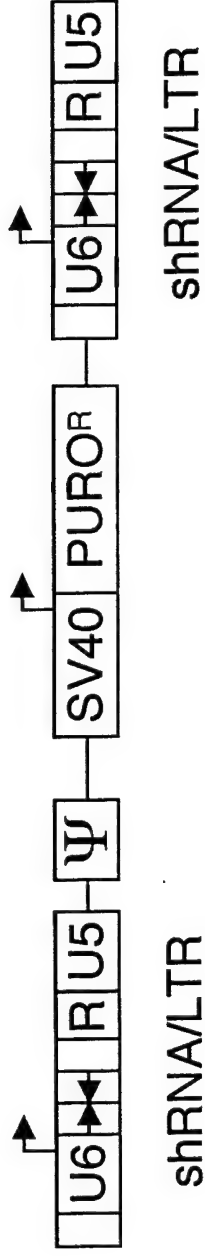
Figure 4: Potential applications of RNAi in mammalians. This figure shows some potential applications of dsRNA RNA triggers of gene silencing in mammals. We envision that both siRNA and expressed shRNAs and siRNAs will have broad utility for genetically manipulating cells both *in vivo* and *in vitro*. Applications may range from finding new drug targets in culture cells to modeling tumor behavior in mouse models, to applying RNAi as a therapeutic tool in the clinic.

Paddison and Hannon, Fig 2

A.

Babe-Puro-LTR-shRNA

| U6 ◄► | R | U5 |
shRNA/LTR

| U5 | R | Ψ | SV40 | PURO^R | U6 ◄► | R | U5 |

shRNA/LTR

B. Murine p53-shRNA

sense strand

```
                            GAA
                               G
GGUCUAAGUGGAGGCCCUUCGAGUGUUA   C
CCGGGUUCACUUCGGGAGGCUCACAGU   GUU
UU
```
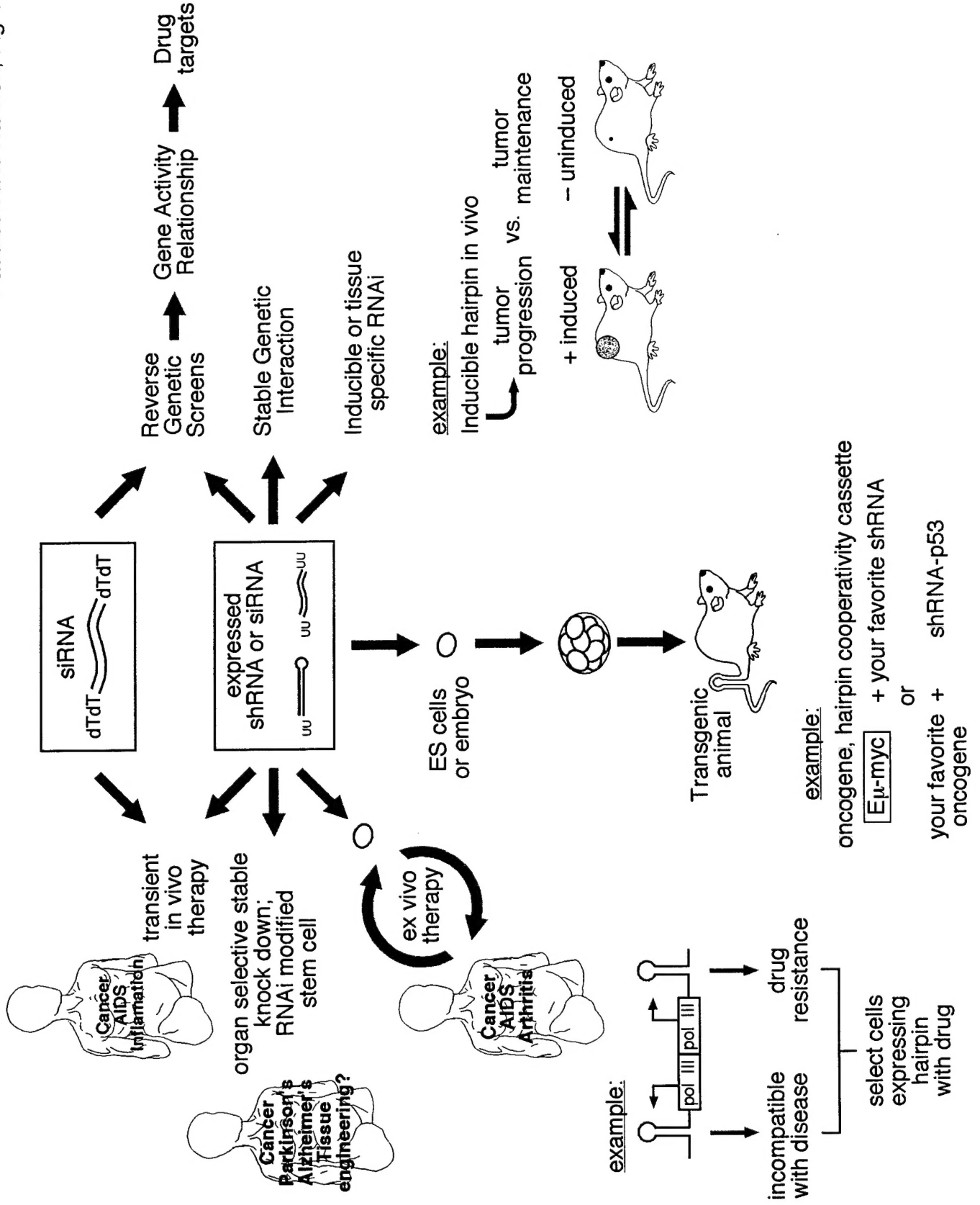
anti-sense strand

C. Mouse Embryo Fibroblasts

Wzl-RasV12

Babe-Puro
shRNAp53

Babe-Puro
control

Paddison and Hannon, Fig 4

1428

Kay

Inhibition of transgene expression in mice

RNA interference is an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence-specific silencing of homologous genes. Here we show that transgene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed *in vivo* from DNA templates. We also show the therapeutic potential of this technique by demonstrating effective targeting of a sequence from hepatitis C virus by RNA interference *in vivo*.

Small interfering RNAs (siRNAs), which mimic intermediates in the RNA-interference (RNAi) pathway, can silence genes in somatic cells without activating non-specific suppression by double-stranded RNA-dependent protein kinase[1]. To investigate whether siRNAs also inhibit gene expression *in vivo*, we used a modification of hydrodynamic transfection methods[2-4] to deliver naked siRNAs to the livers of adult mice. Either an siRNA derived from firefly luciferase or an unrelated siRNA was co-injected with a luciferase-expression plasmid (for construct description and sequences, see supplementary information). We monitored luciferase expression in living animals using quantitative whole-body imaging[5] (Fig. 1a, b), and found that it was dependent on reporter-plasmid dose (results not shown).

In each experiment, serum measurements of a co-injected human α-1 antitrypsin (hAAT) plasmid[6] served to normalize transfection efficiency and to monitor non-specific translational inhibition. Average serum levels of hAAT after 74 h were similar in all groups.

Our results indicate that there was specific, siRNA-mediated inhibition of luciferase expression in adult mice ($P < 0.0115$) and that unrelated siRNAs had no effect ($P < 0.864$; Fig. 1a, b). In 11 independent experiments, luciferase siRNAs reduced luciferase expression (as judged by emitted light) by an average of 81% ($\pm 2.2\%$). These results show that RNAi can downregulate gene expression in adult mice.

As RNAi inhibits the replication of respiratory syncytial viruses in culture[7], we investigated whether RNAi could be directed against a human pathogenic RNA expressed in a mouse, namely that of hepatitis C virus (HCV). (Infection by HCV, an RNA virus that infects 1 in 40 people worldwide, is the most common reason for liver transplantation in the Western [Author: developed?] world.) We fused the NS5B region (non-structural protein 5B,

viral-polymerase-encoding region) of this virus with luciferase RNA and monitored RNAi by co-transfection *in vivo*. An siRNA targeting the NS5B region reduced luciferase expression from the chimaeric HCV NS5B protein–luciferase fusion by 75% (±6.8%; 6 animals per group). This result suggests that it may be feasible to use RNAi as a therapy against other important human pathogens.

Although our results indicate that siRNAs are functional in mice, delivery remains a major obstacle. Unlike siRNAs, functional small-hairpin RNAs (shRNAs) can be expressed *in vivo* from DNA templates using RNA polymerase III promoters[8,9]; they are as effective as siRNAs in inducing gene suppression. Expression of a cognate shRNA (pShh1-Ff1; see supplementary information) inhibited luciferase expression by up to 98% (±0.6%), with an average suppression of 92.8% (±3.39%) in three independent experiments (Fig. 1c, d). An empty shRNA-expression vector had no effect (results not shown); reversing the orientation of the shRNA (pShh1-Ff1rev) insert prevented gene silencing because it altered the termination by RNA polymerase III and generated an improperly structured shRNA. These findings indicate that plasmid-encoded shRNAs can induce a potent and specific RNAi response in adult mice.

RNAi may find application in functional genomics or in identifying targets for designer drugs, offering a better system than gene-knockout mice, as groups of genes can be simultaneously rendered ineffective without the need for time-consuming crosses. Gene therapy currently depends on the ectopic expression of exogenous proteins; however, RNAi may eventually complement this gain-of-function approach by silencing disease-related genes with DNA constructs that direct the expression of shRNAs. Our method of RNAi delivery could also be tailored to take advantage of developing viral and non-viral gene-transfer vectors in a clinical context.

Anton P. McCaffrey*, Leonard Meuse*, Thu-Thao T. Pham*, Douglas S. Conklin†, Gregory J. Hannon†, Mark A. Kay*

*Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, California 94305-5208, USA
e-mail: markay@stanford.edu
†Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA

1. Elbashir, S. M. *et al. Nature* 411, 494–498 (2001).
2. Zhang, G., Budker, V. & Wolff, J. A. *Hum. Gene Therapy* 10, 1735–1737 (1999).
3. Liu, F., Song, Y. & Liu, D. *Gene Therapy* 6, 1258–1266 (1999).
4. Chang, J., Sigal, L. J., Lerro, A. & Taylor, J. *J. Virol.* 75, 3469–3473 (2001).
5. Contag, C. H. *et al. Photochem. Photobiol.* 66, 523–531 (1997).
6. Yant, S. R. *et al. Nature Genet.* 25, 35–41 (2000).
7. Bitko, V. & Barik, S. *BMC Microbiol.* 1, 34 (2001). [Author: only one page?]
8. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. & Conklin, D. S. *Genes Dev.* 16, 948–958 (2002).

9. Tuschl, T. *Nature Biotechnol.* 20, 446–448 (2002).

Figure 1 RNA interference in adult mice. **a,** Representative images of light emitted from mice co-transfected with the luciferase plasmid pGL3-control and either no siRNA, luciferase siRNA or unrelated siRNA. A pseudocolour image representing intensity of the emitted light (red, most intense; blue, least intense) superimposed on a greyscale reference image (for orientation) shows that RNAi functions in adult mice. Annealed 21-nucleotide siRNAs (40 μg; Dharmacon) were co-injected into the livers of mice with 2 μg pGL3-control DNA (Promega) and 800 units of RNasin (Promega) in 1.8 ml PBS buffer in 5–7 s. Mice were anaesthetized and given 3 mg luciferin intraperitoneally 15 min before imaging. **b,** siRNA results (six mice per group) from a representative experiment. Mice receiving luciferase siRNA emitted significantly less light than reporter-alone controls (one-way ANOVA with post hoc Fisher's test). Results for reporter alone and unrelated siRNA were statistically similar. **c,** pShh1-Ff1, but not pShh1-Ff1rev (see text), reduced luciferase expression in mice relative to the reporter-alone control. pShh1-Ff1 or pShh1-rev (10 μg) were co-injected with 40 μg pLuc-NS5B in 1.8 ml PBS buffer. **d,** Average of three independent shRNA experiments ($n = 5$). No error bar is shown for the reporter-alone group as average values for this group are designated as 100% in each of the three experiments. Animals were treated according to the US National Institutes of Health's guidelines for animal care and the guidelines of Stanford University.